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Transmission of Mouse Leukemia by Oral Route[1]

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It has been observed in earlier studies that the mouse leukemia virus could be transmitted with the milk of nursing female mice to their offspring, or to foster-nursed mice [7, 8]; results of these experiments suggested clearly that under certain experimental conditions the mouse leukemia virus could be transmitted by oral route. However, under experimental conditions employed in these previous studies transmission of the virus through the intestinal tract was accomplished in newborn mice; the question remained to be answered whether infection by oral route may also occur in animals beyond the very early infant age.

In an attempt to determine whether experimental transmission of leukemia could be accomplished by oral route in young adult animals, young weanling C3H(f) mice, 3 to 4 weeks old, received a single meal consisting of a ground leukemic cell suspension mixed with milk and bread. As a result, and rather unexpectedly, all animals which ingested the leukemic cell extract developed after several months disseminated lymphosarcomas.

Materials and Methods

Animals. Young, weanling C3H(f) female mice, 3 to 4 weeks old, from our colony were used for this experiment. Several litters were employed and split so that mice in the experimental and in the control groups were litter-mates.

When left undisturbed, mice of the C3H(f) strain bred in our laboratory have a very low incidence of spontaneous leukemia. The rare cases of spontaneous leukemia which developed in untreated C3H(f) mice in our colony have been observed in animals over 12 months old; during the past 10 years the total incidence has not exceeded 0.5 to 1%.

[1] Aided, in part, by grants from the Damon Runyon Memorial Fund and the American Cancer Society.

Preparation of the leukemic extract for feeding. Fragments of thymic lymphomas, leukemic lymph nodes and spleens were removed aseptically from C3H(f) mouse donors with passage A virus-induced leukemia; the tissue fragments were cut with sterile scissors, and ground by hand in a mortar with a small quantity of physiological saline solution added; pasteurized cow milk was then added to obtain a cell suspension of approximately 20% concentration.

Voluntary ingestion of a meal containing leukemic cell extract. The leukemic cell extract was then poured over small pieces of bread and placed in an open sterile petri dish on the bottom of a cage containing a group of 12 young adult C3H(f) mice; the mice, which had been kept without food and water for the preceding 20 h, fed voluntarily on the experimental meal containing the leukemic cell suspension. This was the only treatment these mice received. Seven hours after this treatment the mice received their usual daily meal consisting of Purina Laboratory Chow pellets and tap water; twice a week they also received oats and fresh lettuce. They were kept for observation without any further treatment.

Control Group. The control group consisted of 8 C3H(f) female mice, 3 to 4 weeks old, litter-mates of those employed in the experimental group. The 8 mice in the control group were left for 20 h without food and water; after this lapse of time a leukemic cell suspension, heated to 56°C for 1 hour and mixed with pasteurized cow milk and bread, was placed in an open petri dish at the bottom of their cage. Seven hours later the control mice received their regular daily food consisting of Purina Laboratory Chow pellets and tap water; they also received twice weekly oats and fresh lettuce. They were kept for observation without any additional treatment.

Results

The results are summarized in table I. Twelve mice in the experimental group ingested voluntarily a single meal consisting of fresh leukemic cell suspension mixed with milk and bread, and all developed generalized leukemia at ages varying from 4 to 12 months, i.e. 3 to 11 months after the ingestion of the virus-containing meal. All 12 animals which developed leukemia in this group developed disseminated

Table I. Transmission of mouse leukemia by oral route

	No. of mice inoc.	No. dev. leuk.	Leuk. incid.	Avg. age leuk. dev. mos.
Experimental group	12	12	100	7.6
Controls	8			

Virus inoculation consisted of a voluntary ingestion of a meal containing leukemic cell suspension in bread and milk. The control animals received heated (56°C for 1 hour) leukemic cell suspension with bread and milk. All mice were 3 to 4 weeks old at the time they received virus.

Age range: leukemia developed 4 to 12 months.



Figure 1 This C3H(f) female mouse No. 541 ingested, when 3 weeks old, leukemic cell suspension mixed with bread and milk; the voluntarily ingested meal was the only treatment this mouse received. Three months later at the age of 4 months, this mouse developed generalized lymphatic leukemia (WBC 61 400/mm³ with 48% of lymphoblasts in peripheral blood). Note the very large thymic tumor large spleen, large oblong mesenteric tumor and enlarged inguinal, axillary and cervical lymph nodes.

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lymphosarcomas involving predominantly the thymus, liver spleen, and peripheral lymph nodes (fig 1) Electron micrographs of virus particles from a thymic lymphoma are shown in figure 2. Their peripheral blood counts ranged from 11 000 to 94 600 WBC/mm³ The predominant white blood cells were of the lymphocytic series the relative incidence of lymphoblasts in peripheral blood observed in 9 of the 12 leukemic animals ranged from 2 to 51 Most of these mice had also a moderate to moderately-severe anemia.

In the control group, all 8 mice remained in good health they are now 15 months old.

Discussion

Even though only a small number of animals was employed in the experiment described in this preliminary report, the results obtained suggest clearly that under certain experimental conditions, using a potent leukemogenic virus, mouse leukemia can be transmitted by oral route by a single, voluntarily ingested meal, to 3 to 4-week-old mice of a susceptible inbred line Thus far we have not observed a similar virus transmission following feeding of young weanling C3H(f) mice with a single meal containing a leukemic cell suspension prepared from organs of Ak mice with spontaneous leukemia, mixed with bread and milk however this experiment is still in progress, and positive results may be obtained at a later date. It is quite possible, nevertheless, that a more potent passaged virus may be needed to transmit leukemia by oral route. An experiment dealing with an attempt to transmit mouse leukemia by feeding of passage A virus filtrate, instead of leukemic cell suspension, mixed with milk and bread, is also in progress.

Although we have employed a leukemic cell extract for the preparation of the experimental meal in this study it is reasonable to assume that transmission of the disease was accomplished by a virus released by the leukemic cells present in the ingested food. Leukemia which developed in the experimental mice appeared after a rather long latency varying from 3 to 11 months, and was generalized involving thymus, spleen, and peripheral lymph nodes in all animals it is unlikely that leukemic cells would have survived and that they became implanted somewhere along the intestinal tract after oral ingestion furthermore, had the transmission been caused by accidental implantation of leukemic cells ingested with the meal one would have rather expected a shorter latency and the development of lymphoid tumors

at the site of implantation somewhere along the intestinal tract, with generalization of the disease occurring only as a delayed and secondary manifestation.

We have demonstrated in previous studies that under normal life conditions the common pattern of virus transmission in mouse leukemia follows the vertical route, from parents to offspring directly through the germinal cells [5 6] The observation that the mouse leukemia virus can induce disease following a single administration by oral route suggests that under certain experimental conditions, employing a potent passage virus, transmission may not necessarily be limited to a vertical route, but may also occur in the more conventional manner commonly observed in many bacterial and viral diseases Whether *this may occur under natural life conditions, however remains to be* determined.

Transmission by oral route thus far accomplished required a highly potent passage virus and highly susceptible mice These are rather artificial laboratory conditions which may not be readily duplicated in nature. On the other hand, the result of this experiment may also imply that under certain limited conditions transmission of leukemia by oral route should be considered as a possibility not only in mice but in other species also This has been previously recognized in studies dealing with natural transmission of chicken lymphomatosis [1 2]

Digestive System Site of Entry and also of Multiplication of the Mouse *Leukemia Virus*

The observation that effective virus infection leading to the development of disease can be obtained in mice following voluntary ingestion of a single meal containing a leukemic cell extract is of considerable interest it indicates that the mucous membranes of the digestive tract are susceptible to virus penetration, and that the virus may resist inactivation and destruction by the secretions of the digestive system.

Electron microscopic studies carried out in our laboratory in cooperation with Dr D G Feldman *on C3H f) mice injected, when* newborn, with the passage A virus (Gross) demonstrated that the mouse leukemia virus can multiply and accumulate in the salivary glands and in the pancreas (fig 3) [3, 4] The development of virus particles by the process of budding at the edge of cytoplasmic membranes of the epithelial cells was observed in both organs the virus

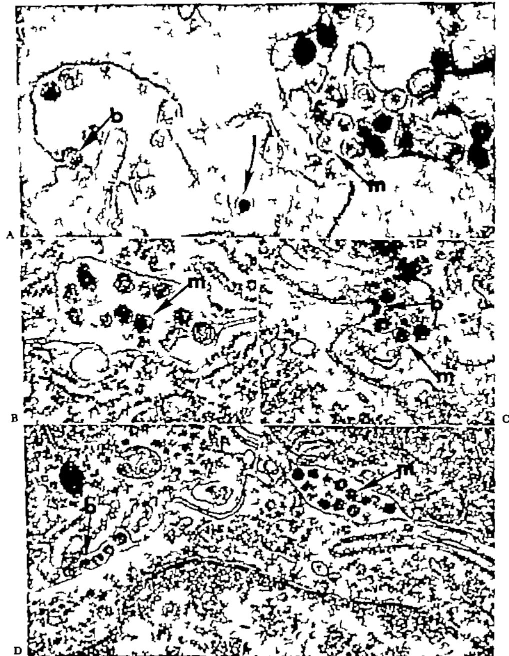


Fig 3. Electron micrographs of ultrathin sections prepared from organs of the digestive system of C3H(f) mice in which leukemia was induced by routine intramuscular inoculation of the passage A mouse leukemia virus filtrate. (A) *Salivary gland:* Virus particle (b) budding from epithelial cell and doughnut-like immature (I) and mature (m) type C virus particles, with nucleoids, in intercellular space (30,000 ×). *(B) Pancreas.* Type C virus particles (m) in intercellular space between 2 pancreatic acinar cells (42,800) *(C) Duodenum.* Virus particle budding (b) from epithelial cell and mature type C virus particles (m) in inter cellular space (42,800). *(D) Colon:* Virus particle budding (b) from epithelial cell and mature type C virus particle (m) in intercellular space (33,600)

was then released into the intercellular spaces. Virus particles were also found in the ducts of the salivary glands and of the pancreas.

In more recent electron microscopic studies replication of the virus and formation by the process of budding was also observed in the intestinal tract, particularly in the epithelial cells of the duodenum, jejunum, and colon of virus-injected C3H(f) mice

Summary

Three to four week-old C3H(f) female mice received a single meal consisting of passage A virus-induced leukemic cell suspension mixed with milk and bread. The meal was not forcibly fed, the mice consumed this mixture voluntarily All 12 mice (100%) developed leukemia after latency varying from 3 to 11 months. In control group 8 C3H(f) mice ingested a single meal consisting of heated (56°C for ½ hour) leukemic cell suspension mixed with milk and bread. All mice in the control group remained in good health; they are now 15 months old. Electron microscopic studies of organs of C3H(f) mice that had been inoculated routinely with passage A mouse leukemia virus revealed the presence of clusters of characteristic virus particles, and multiplication of virus particles by budding, in salivary glands, in pancreas, and also in epithelial cells of the duodenum, jejunum and colon.

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Isolation of Mycoplasmas in Leukemia and Other Diseases[1]

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The conflicting data reported by a number of investigators on the relationships between Mycoplasmas and leukemias, malignancies and other systemic diseases, together with our previous studies on the etiological role of these microorganisms in the pathogenesis of some human diseases, called our attention to the possibility of isolating these agents from leukemias and related diseases.

Positive isolations of Mycoplasmas from leukemic materials have been reported by Murphy *et al.* [23 24] They were able to isolate Mycoplasmas in nearly 50% of the leukemic specimens tested. Higher incidence of isolation has been found after passages of the specimens in cell cultures. A fairly high incidence of isolation (40%) has also been reported by Barile *et al.* [6 7] On the contrary a very low incidence of positive isolation has been reported by Hayflick and Koprowski [17] Hayflick and Stanbridge [18] and by Dreyer [12] Simkovics [29] obtained negative results in all of the leukemic cases studied.

It has to be pointed out that all these attempts have been done by direct plating on acellular media, so that the possibility that the isolates could be derived from latently infected cell cultures used for assay rather than from the clinical specimens has been rouled out. Nevertheless, earlier studies on the isolation of Mycoplasmas in tissue cultures infected with leukemic materials have been reported by Grace *et al.* [15] by Negroni [25] and by Daldorff and Bergamini [8, 9]

This work is a part of the research program worked out, since many years, in our laboratories, on the possibility to isolate viruslike agents

[1] This work has been conducted with grant of CNR (Gruppo Nazionale di Medicina Sperimentale, Sezione Virologia).

from leukemic patients and to study their relationships with human leukemia.

Materials and Methods

The media employed in these studies for the isolation of Mycoplasmas consisted basically of Difco PPLO agar or broth at a concentration of 3.4%, to which the following ingredients were added to enrich them according to MCGARRITY *et al.* [23]: Brain hearth infusion (3.7%) human or agamma-horse serum (final concentration 20%) baker yeast extract (25%) 3% AGVD stock solution (per 100 ml 2.1 g of arginine 3.0 g of glutamine, 5.0 g of dextrose 10 ml of Eagles 100 × basal Vitamins). The final pH of the media has been adjusted to 7.4–7.6. We have not still done attempts to isolate Mycoplasmas in media at a lower pH as indicated more recently by some authors [24, 18], on the basis of the observation that most Mycoplasmas prefer alkaline conditions.

Bone marrow specimens obtained by sternal puncture from leukemic and non leukemic patients were directly inoculated into liquid media at 1:20 dilution within 1 to 3 h after collection. The samples were then incubated at 37°C in microaerophilia (95% N_2, 5% CO_2).

Each material received 2–3 successive passages on fluid and solid media at intervals of 4 to 6 days, before being considered negative.

Two out of four Mycoplasma strains isolated were classified trough the complement fixation test. The technique employed both in preparing complement-fixing antigens (used, in this case, not inactivated) and in performing the reaction has already been described [1,2].

Thirty two cases of leukemia have been examined (9 cases of chronic myelogenous leukemia, 6 cases of chronic lymphatic leukemia, 17 cases of acute leukemia). Bone marrow specimens were taken from the patients in three different stages in the course of the disease-usually when the initial symptoms appeared, during remission (clinical and hematological) and during relapse of the disease. In parallel to the leukemic specimens, 9 cases of malignancies of the lympho-reticulosarcomatous type, 16 cases of hemopathies other than leukemia, 4 cases of collagen diseases and 12 cases of various pathological conditions have been studied (table I).

Results

So far only four strains of Mycoplasma have been isolated. Of these, two were isolated from two cases of acute leukemia. One of these strains showed tiny colonies on agar and slight growth capacity. It has been lost after two passages in cell-free media. The second one, which was reisolated on two occasions from two different marrow specimens from the same patient, was serologically related to *Mycoplasma fermentans* as shown by the complement fixation test (table II).

The other two isolated strains were obtained from a case of a pure erythroblastic aplasia and from a case of hypochromic hyposideremic anemia, respectively. The strain isolated from erythroblastic aplasia has also been lost after few serial passages in cell-free media. This strain showed on agar colonies of medium size with a center well differentiated from the periphery. The other strain was found to be similar to *Myco-*

Table I Attempts to isolate Mycoplasmas from leukemias and other pathological conditions

Diseases	No. of cases	No. of passages performed	No. of strains isolated	Characteristics of the isolated strains
Acute leukemia	17	3	2	*L.M.V.* tiny colonies strain, lost in serial passages -*C.E.* strain serologically related to *M. fermentans*
Chronic myelocytic leukemia	9	3	0	
Chronic lymphocytic leukemia	6	3	0	
Marrow aplasias	4	2	1	-RI large colonies strain, lost in serial passages
Anemias (of various type)	7	2	1	TAM.A. strain serologically related to *M. orale*
Plasmocytoma	5	2	0	
Hodgkin's disease	5	2	0	
Lympho- or reticulosarcoma	4	2	0	
Collagen diseases	4	2	0	
Epitelial neoplasms	2	2	0	
Other conditions	12	2	0	

Spleen diseases, hepatic cirrhosis, renal diseases, etc.

plasma orale both for the characteristics of the colonies and for the serological behaviour as shown by the complement fixation test.

Discussion and Conclusions

The data reported here show that Mycoplasmas could be isolated from bone marrow specimens obtained from leukemic and non leukemic patients in very low percentage. Furthermore, it appears to be no differences in the rate of isolations between leukemic and control materials It has also to be pointed out, however that the positive

Table II. Serological characterization of Mycoplasmas isolated from C. E. (*acute leukemia*) and from TAM.A. (hypochromic anemia)

Sera	Complement fixation test Mycoplasma antigen isolated from C. E.	Mycoplasma antigen isolated from TAM.A.
Patient's serum	<10/<10	<10/<10
Anti *Mycoplasma pneumoniae* (FH)	10/10	<10/<10
Anti *Mycoplasma fermentans* (PG 18)	40/20	<10/<10
Anti *Mycoplasma hominis* 1 (PG. 21)	10/10	<10/<10
Anti *Mycoplasma hominis* 2 (PG. 27)	10/10	<10/<10
Anti *Mycoplasma orale*[1] (Patt)	–	80/40
Anti Mycoplasma P[2]	10/10	<10/<10

All the antisera, except those from patients, have been previously absorbed (1 h at 37° C, 18 h at 4 C, successive centrifugation 2 h at 15,000 rpm) with normal antigens (normal culture medium). The numerator gives the reciprocal of the highest antigen dilution yet positive the denominator the reciprocal of the highest serum dilution yet positive.

From Robbin Lab., Cleveland, N.Y All other sera have been prepared in rabbits [1 2].

P strains have been isolated from latently infected amniotic human cells (as shown by positivity of BARILE and SCHIMKE's argininedehidrolase test) when these cell cultures were inoculated with chick embryo materials infected with human leukemic marrows and had shown a clear CPE.

isolation from the case of erythroblastic aplasia cannot be considered as an isolation from a control material owing to the hemato-chemical characteristics of this patient (monoclonal gammopathy disorder)

It seems to us that the low rate of isolation cannot be due to eventual inadequacies of the methods used. In fact, using the same isolation procedures, Mycoplasmas could be isolated in MURPHY's Laboratories with high incidence from leukemic patients. Furthermore, in our laboratories, Mycoplasmas have been isolated from the upper and lower respiratory and from genitourinary tracts with relatively high incidence[1]

It must be also remembered that, when, in successive studies, the color changing techniques were adapted to the studies of Mycoplasma

It has to be pointed out, however that we were unable to propagate in cell-free media three *PPLO strains* (K[illegible], K[illegible], Z[illegible]) *received* through the courtesy of Dr MURPHY Only with the K[illegible] strain, it was possible to observe some colonies during the first passage on agar All further attempts to propagate this strain were unsuccessfull. This indicates that it is possible to get variable results in different laboratories. Another explanation could be the progressive inactivation the strains underwent to during shipment of the materials to our laboratories.

growth in cell-free media (glucose and phenol red as an indicator for the growth of *M. Pneumoniae* and *M. fermentans* arginine and chresol red for large colonies Mycoplasma strains which metabolize this aminoacid) we could often note a lack of correlation between the growth in fluid media, which was positive, and the growth on agar negative. This means that the same test has to be used also in the studies of isolation of PPLO from leukemic materials before the problem can be definitely settled out.

The easy loss of the isolated strains during serial passages further strengthens the idea of the difficult adaptation of the new isolated strains to the cell-free media. The two isolated strains which we were able to propagate in serial passages turned out to be serologically related to *M. fermentans* and *M. orale*, respectively.

However no correlation has been found between isolated strains and presence of antibodies in the blood of the patients from which the strains were isolated. These results agree with those of BARILE [6] and MILLIAN and SJOLEND [22] and are in contrast with those of MURPHY *et al.* [24].

From these results a critical conclusion can be drawn on the frequency and the significance of the isolation of Mycoplasmas from leukemias and other malignancies in human pathology. The results obtained by various authors, together with the number of their observations and several of the methodological peculiarities, are summarized in table III. First of all, it appears that the frequency of isolation of Mycoplasmas from tissue of leukemic patients and of patients suffering for other malignancies is higher than that observed in control subjects.

MURPHY *et al.* [24] affirm that Mycoplasmas may cause active infections in leukemic patients, presumably because of decreased host resistance during progression of the disease. They say that Mycoplasmas may play a role in the pathogenesis of leukemia and contribute to its clinical course inciting hyperplastic changes in the spleen, lymphnodes, thymus, bone marrow and also a febrile response and possibly toxemia.

On the contrary SABIN [27] HUEBNER (personal communication) and LECLERC and SILVESTRE [20] consider Mycoplasmas isolated from leukemic tissues as contaminants in patients with a disease which severely impairs host defenses against all the types of infections. The results of most authors and our results seem to support more strongly this last interpretation especially when considering the general

Table III

Authors	Source and number of isolates	Isolation method	Name of the strains	Serological type[1]
GRACE *et al* [15]	Leukemic spleen (4/21)	Culture on various cell lines	880	*M. mergenhagen* (*M. pulmonis*)
NEGRONI [23]	Leukemic marrow (10/10)	Human kidney embryo cells		*M. pulmonis*
DALLDORF and BERGAMINI [8]	Burkitt sarcoma	Human kidney embryo cells		Not classified
ARMSTRONG *et al* [4]	2 human tumors	Neoplastic cells		*M. orale*?
MURPHY *et al* [25, 4]	Leukemic bone marrow 50/700; 13 directly on agar (in the whole 30% of isolation with their actual techniques)	Amnion and kidney human cells or directly in acellular media	F_{11}, F_1; K, K_{26}, Z_{44}	*M. hyorhinis*; related or not to *M. fermentans*[2]
HAYFLICK *et al* [17, 18]	Leukemic bone marrow (1/100)	Directly on acellular media	N_1	
BARILE *et al* [6, 7]	Leukemic bone marrow (4/10)	Directly on acellular media	19, 274, 278, 81	*M. orale*
DMOCHOWSKI *et al* [27]	Leukemic bone marrow (1/10)	Directly on acellular media		*M. orale*[3]
SOMERSON [29]	Leukemic bone marrow (0/10)	Directly on acellular media		
RABIN [27]	Human lymphoma	HEP cultures; primary human kidney cells		*M. hyorhinis*
ALTUCCI *et al* [3]	Leukemic bone marrow (2/32)	Directly on acellular media	LM ... Q, I	*M. fermentans*[4]

[1] Many of reported serological characterizations have been defined by the work of PURCELL *et al* [26].
[2] The three signs represent the main serological groups of Mycoplasmas isolated by MURPHY: K_{26} from undifferentiated sarcoma closely related to *M. fermentans*; K_{44} from acute leukemia distantly related to *M. fermentans*; Z_{44} from myelomonocytic acute leukemia not related to *M. fermentans*. It must be remembered that MURPHY *et al* have obtained positive isolation also with much lower percentages, from control sera [24].
A strain (N_1) has been recently isolated in acellular media from a vesical papilloma.
[3] The first three strains have been identified as *M. orale*; the last has not been classified.
[4] The mother of the two strains has not been classified because of the loss in serial passages. It does not result a number difference in the Mycoplasma isolation from leukemias and other diseases (see text).

saprophytism of Mycoplasma species isolated from leukemias with frequencies which also seem partially related with the different working laboratories.

Nevertheless, it has to be remembered that phenomena of cellular transformation by Mycoplasmas have been reported [21] similarly to what happens with *Polyoma virus* Anyway the general significance of cellular transformation has not yet been fully understood. Furthermore, it is also well known that non oncogenic viruses (i. e. Myxoviruses [28]) may in particular conditions, determine the same phenomenon.

Among the afore mentioned controversies it has also to be remembered that some authors consider Mycoplasmas either as secondary invaders, eventually capable to display a pathogenic action [15 16] or as agents capable to exercise, in association with viruses, a pathogenic synergism [20] or finally as agents that might play a helper' role in conjunction with other entities (viruses) [15]

These studies and interpretations open very interesting problems considering the variety and the discrepancies of the results obtained by various authors, it seems to us suitable to remain in a waiting position which does not necessarily imply a complete autogenomy between Mycoplasmas and viruses in the etiology of human leukemia. In order to clarify further this problem, we think that investigations on the parallel isolation of both Mycoplasma and viruses from the same materials have to be worked out. Researches on this line are now in progress in our laboratories.

During the publication of this work, Smith *et al.* (Arch. ges. Virusforsch. 20 294 1966) have also reported negative results as far as concerns the isolation of Mycoplasmas from 107 leukemic patients.

Summary

Attempts have been made t isolate in acellular media Mycoplasmas from bone marrow specimens of patients both leukemic and suffering for other diseases.

Two Mycoplasma strains were isolated from two acute leukemia cases of 32 studied one, showing minute and tiny colonies on agar growth, was lost in successive passages; another was serologically related to *Mycoplasma fermentans* and it was isolated twice from two different marrow samples.

Other two Mycoplasma strains (large colonies Mycoplasma *Mycoplasma orale*) were isolated from two cases of erythroblastic plasia and hypochromic anemia, respectively

The data reported show that Mycoplasmas could be isolated from bone marrow specimens obtained from leukemic and non leukemic patients in very low percentage. Furthermore it ppears to be no differences in the rate of isolations between leukemic and non leukemic materials.

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The *in vitro* Uptake of ^{35}S L-Methionine by Normal and Leukaemic Leucocytes

KATHRINE M. SLOANE and J. M. BRIDGES

The present paper reports some observations on the *in vitro* uptake of methionine labelled with ^{35}S and its incorporation into protein by leucocytes from normal subjects and patients with various types of leukaemia. This study followed from the observation that patients with leukaemia had an increased level of methionine in the urine [5]. One of the important biological functions of this amino acid is to act as a methyl group donor in the metabolism of vitamin B_{12} [9] and studies were carried out on the influence of the level of this vitamin on the methionine uptake. It has been found that subjects with chronic myeloid leukaemia have an increased serum level of vitamin B_{12} [3, 6, 10] and their leucocytes contain more S-adenosyl methionine than do those of normal subjects [2].

Materials and Methods

Healthy laboratory personnel served as control subjects.

Twenty patients with leukaemia, all but one of whom were adults, were studied; of these 8 had acute, 6 had chronic myeloid and 6 had chronic lymphatic leukaemia. Blood from these subjects was tested on many occasions, at various stages of their illness, and while they were being treated with a variety of chemotherapeutic agents.

Leucocyte suspensions were prepared from blood anticoagulated with EDTA (10 ml blood to 0.2 ml 10% dipotassium sequesteric acid), by incubation at 37°C for approximately 60 min. The supernatant plasma was harvested and the leucocyte count determined. This was adjusted either by dilution, with autologous leucocyte free plasma, or concentration by centrifugation, *so that it lay within the range of 7,000 to 12,000 cells/mm³*.

One ml of this suspension was incubated with 0.5 ml 0.135 µg/ml non-radioactive methionine and 0.1 ml ^{35}S methionine (0.05 mc/100 ml) in a stoppered, graduated 10 ml tube for 120 min at 37°C. The reaction was then stopped by immersing the reaction vessel in ice cold water for 5 min. The leucocytes were then separated by centrifugation at approximately

300 g for 10 min and the supernatant discarded. The leucocyte button was mixed with 5 ml ice cold distilled water 30 sec later 5 ml EDTA iminazole buffered saline (10 g sequestenic acid and 3 g iminazole in 1 l, 0.9% saline) was added; this caused lysis of the remaining red cells. The cells were again deposited by centrifugation and washed with 10 ml buffered saline (10 g EDTA and 3 g iminazole in 2 l, 0.9% saline) after which they were resuspended in 1 ml of this solution.

After incubation and washing, an aliquot of the final suspension was removed for a white cell count and 0.2 ml was placed in counting vial with 10 ml scintillation fluid (5 g PPO, 2 5-Diphenyloxazole, 0.3 g Dimethyl POPOP 1 4-1 [2-(4-Methyl-5-Phenyl-oxazolyl)] Benzene, 750 ml Toluene and 250 ml Ethanol) and 0.5 ml hydroxide of hyamine. The radioactivity was counted in Packard Tricarb scintillation counter

With each experiment a high standard (0.1 ml ^{35}S methionine, 0.5 ml hydroxide of hyamine and 10 ml scintillation fluid) low standard (0.1 ml non-radioactive methionine, hyamine and scintillation fluid, as above) and blank (0.5 ml hyamine and 10 ml scintillation fluid) were counted. In the calculation of results from counts thus obtained, allowance for quenching effect was omitted, since the composition of the contents of each vial were similar

The results were expressed as pg of methionine taken up per million cells. These were calculated using the formula

$$0.0135 \times \text{counts/minute of suspension} \times 10^6 \Big/ \left\{\begin{matrix}\text{counts/minute} \\ \text{for high standard}\end{matrix}\right\} \times \left\{\begin{matrix}\text{white cell count/ml} \\ \text{of suspension}\end{matrix}\right\}$$

In order to establish the optimum conditions for the uptake of methionine by human leucocytes, the cells were incubated at temperatures of 4° C, 20° C, 37° C and 56° C. This, and an experiment in which the reaction was carried out in the presence and absence of 1 drop of formalin, were also performed to illustrate that the uptake of ^{35}S methionine is an active process.

T show the effect of added vitamin B_{12}, the above reaction was carried out on cells which had been incubated with 0.1 ml cyanocobalamin (0.5 μg/ml) for 60 min at 37° C prior to the addition of methionine.

I experiments in which the incorporation of ^{35}S methionine into the protein of the cells was studied, 0.2 ml of the final white cell suspension was added to 1 ml of 15% ice cold trichloroacetic acid (TCA) and the resulting precipitate separated off by centrifugation at pproximately 1500 g for 10 min.

The precipitate was washed in 1 ml of fresh ice cold TCA and then in 1 ml of ethanol before being dissolved in 1 ml hydroxide of hyamine and 10 ml scintillation fluid. This was treated similarly to other radioactive samples for counting.

Vitamin B_{12} levels were measured using Lactobacillus leichmannii and folic acid levels were measured by the method of WATERS and MOLLIN [11].

Results

The uptake of ^{35}S methionine in pg/million cells by the leucocytes from normal subjects and those with acute, chronic myeloid and chronic lymphatic leukaemia is illustrated in figure 1 For the normal subjects this varied from 5.5 to 21.3 pg/million cells. These subjects all had whole blood white cell counts, serum folic acid and vitamin B_{12} levels within the normal range and there was no correlation between the uptake of methionine and these parameters.

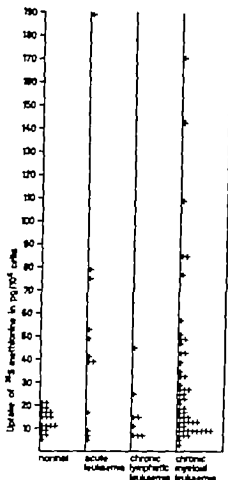


Fig 1 Scattergram illustrating the uptake of ^{35}S methionine by normal and leukaemic leucocytes.

One of the subjects with acute leukaemia was in complete remission at the time of study and the uptake by his white cells was 7 0 and 4 7 pg/million cells, tested on separate occasions. In the remaining subjects with acute leukaemia the uptake varied from 9.2 to 328.0 pg/million cells (table I) There is no obvious correlation between the level of methionine uptake and the peripheral blood leucocyte count in our subjects with acute leukaemia.

The cells from our patients with chronic lymphatic leukaemia exhibited an uptake mostly within the normal range, there being only two which showed a slightly raised methionine uptake. As in the case

Table I. Whole blood leucocyte count compared with uptake of ^{35}S methionine by cells from patients with acute leukaemia

Subject No.	Leucocyte count/mm	Uptake pg/million cells
1	5,600	7.07
	4,400	4.74
2	22,000	9.27
	90,000	29.56
3	–	29.0
	1 150	75.31
4	82,000	328.1
5	–	18.95
	76,000	188.5
	5,800	79.87
6	27,000	20.63
7	16,000	22.1
8	64,000	32.74

Table II. Whole blood leucocyte count compared with ^{35}S methionine uptake by cells from chronic lymphatic leukaemia patients

Subject No.	Leucocyte count/mm	Uptake pg/million cells
9	304,000	6.9
10	35,600	11.7
	30,500	15.0
11	48,400	17 4
	8,000	24.7
12	11 400	7.9
13	5,100	14.2

of the acute leukaemics, no relationship could be observed between the peripheral blood white cell count and methionine uptake (table II)

The uptake, which in each individual with chronic myeloid leukaemia was tested on several occasions, varied greatly (table III) This uptake did not correlate with the serum vitamin B_{12} and folic acid levels, nor with the white cell count of the whole blood, nor could it be related to any pattern in regard to the patient s clinical state or therapy

Table III. Chronic myeloid leucocyte uptakes and their correlation with whole blood leucocyte count B_{12} level and folic acid level

Patient No.	Leucocyt count cells/mm	Uptake pg/million cells	B_{12} pg/ml	Folic acid ng/ml
15	–	24 7	1,080	4 4
	13 700	13.7	2,742	6.5
	10,800	13.43	1 164	6.4
	11 400	16.87	1,269	3.3
	9,800	17.68	1,371	8.0
	–	14 19	1,227	9.2
16	13,900	7 16	7,000	5.8
	11,200	9.78	2,619	–
	10,200	–	1,800	8.3
	7 600	8.8	>3,000	3.7
17	29,800	6.38	>3,000	4.2
	30,000	6.61	3,000	6.2
	–	24.49	–	–
	14 400	18.79	4,500	3.1
	16,300	27 7	6,300	
18	19,900	31.7	343	4.5
	–	33.0	843	13.9
	21,300	32.19	367	8.7
	26,500	32.99	732	8.0
19	15,100	3.28	1,098	4.3
	17,300	11.08	–	–
	20,800	10.91	1,980	5.5
	24,400	13.86	1,575	2.4
20	13,500	84 7	1,920	4.5
	14,200	4.37	2,772	4.2
	–	6.82	2 725	4.5
	13 000	22.64	3,075	3.5

The relationship between the incubation temperature and methionine uptake is shown in table IV A considerable variation occurs in the methionine uptake at different temperatures, with an optimum uptake at 37°C.

When the uptake test was performed on cells fixed with formalin and on live cells from the same population (table V) the live cells gave normal readings but the fixed cells gave results which were very similar to that of the low standard.

The results of the experiment on the incorporation of methionine into the proteins of the white cells indicate that approximately 30%

Table IV Effect of incubation temperature on uptake of methionine

Subject No.	Uptake, pg/million cells			
	5°C	21°C	37°C	55°C
Normal				
14	–	7.99	21.28	13.04
Chronic myeloid leukaemia				
15	3.10	7.32	12.03	–
16	1.95	5.84	10.91	–
17	8.97	13.96	17.00	–
18	2.75	5.88	24.49	–
19	2.52	5.94	13.45	0.60
Mean	*3.85*	*7.79*	*15.57*	*0.60*
Acute leukaemia				
21	6.70	15.01	29.56	0.39
22	5.58	18.01	29.00	–
Mean	*6.14*	*16.51*	*29.28*	*0.39*

Table V Comparison of ^{35}S methionine uptake by live leucocytes and formalin fixed cells from the same population

Uptake by live cells pg/million cells	Uptake by fixed cells pg/million cells
6.929	0.864
5.005	0
5.13	0.7
6.17	0.02
3.76	0.04

of the methionine taken up by the white cells is incorporated into the protein within the two hour incubation period (table VI)

In table VII is set out the uptake of methionine following the addition of vitamin B_{12}

Discussion

The method employed in the present work was very similar to that used by Weisberger *et al.* [13] who found the methionine uptake by normal leucocytes to be 23 100 counts/min/ml packed leucocytes.

Table VI. Uptake of methionine ^{35}S by white cells compared with its incorporation into protein by cells of the same population

Test No.	Uptake pg/million cells	Incorporation into protein pg/million cells	% total uptake incorporated in protein
1	84.07	28.02	33.2
2	109.53	35.74	32.8
3	8.456	2.579	30.2
4	9.56	3.073	32.5
5	21.28	5.81	27.4
6	8.92	2.27	25.5
7	9.33	2.93	31.4
8	18.2	5.67	31.1
9	4.0	1.14	28.5

Table VII. Comparison between uptake of ^{35}S methionine by leucocytes in presence or absence of added vitamin B_{12}

Test No.	Uptake without added B_{12}	Uptake with added B_{12}	Serum B_{12} pg/ml
Normals			
1	12.40	15.93	396
2	10.58	12.25	392
3	9.73	12.29	–
4	15.28	16.44	519
5	6.86	7.84	374
Acute leukaemia			
1	9.48	8.03	597
2	11.71	11.68	156
3	19.25	18.60	>600
4	15.34	14.73	–
5	16.82	15.70	876
6	16.40	15.39	–
Chronic lymphatic leukaemia			
1	7.07	7.89	2,790
2	19.42	13.02	–
3	7.89	8.05	1,863
4	13.79	14.76	259
Chronic myeloid leukaemia			
1	6.15	10.64	3,000
2	10.51	11.89	–
3	27.72	28.22	–
4	12.09	15.33	2,742
5	3.76	5.03	2,772
6	5.83	7.38	3,000
7	8.78	10.78	2,619

Using appropriate data given by these authors, this would be equivalent to approximately 10.01 pg methionine/million cells, a result very similar to ours. At 37° C the uptake was definitely greater than at the other temperatures, not only for normals, but also for subjects with leukaemia. WEISBERGER and LEVINE [12] also found 37° C to be the optimum temperature for methionine uptake.

When formalin was added to the leucocyte suspension prior to the addition of methionine, the uptake of methionine was greatly impaired. These results would suggest that under the conditions of these experiments the uptake of methionine is predominantly due to an active transport process. HELMREICH and KIPNIS [4] studied the uptake, by lymph node cells, of an amino acid analogue, which has a common transport system with methionine and found that the mechanism of uptake was dependent on the concentration of the amino acid.

Our subjects with acute and chronic myeloid leukaemia showed different patterns of methionine uptake to that of the control subjects, whereas those with the chronic lymphatic type of disease were essentially similar to the controls. The subjects with acute leukaemia, not in complete remission, showed a higher than normal uptake and in one instance of a child with acute leukaemia, this was of the order of 15 times greater than that shown in any control.

In patients with chronic myeloid leukaemia we found that the level of uptake tended to be higher than normal, but the most striking feature was the variation, not only from patient to patient, but in the same patient tested on separate occasions. This could not be correlated with the clinical status, therapy or peripheral blood leucocyte count. In the majority of our patients the vitamin B_{12} level was greatly raised, being similar to those reported by other authors [3, 6, 10] However the B_{12} level could not be associated with any pattern in methionine uptake and the addition of this vitamin *in vitro* had no effect on the degree of methionine uptake.

We studied the incorporation of ³⁵S methionine into protein in nine of the subjects under observation and correlated this with their level of methionine uptake. The incorporation in all cases studied, when expressed as a percentage of the uptake, showed a remarkably constant relationship being in all cases very close to 30% Other authors have studied the incorporation of amino acids into the protein of leucocytes, but none appear to have observed a direct relationship between the amount taken into the cell from the surrounding medium and the proportion of this incorporated into protein [1 7 8]

The phenomenon of uptake and incorporation of amino acids by leukaemic leucocytes may be of practical import in the near future, because of the demonstration that the sensitivity of the leukaemic cell to treatment with L-asparaginase may be predicted from its 'handling' of amino acids.

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Summary

The *in vitro* uptake of ^{35}S methionine by leucocytes from 15 control subjects and 20 subjects with leukaemia was studied. Leucocytes from patients with acute and chronic myeloid leukaemia had greater than normal uptake and for the subjects with chronic myeloid leukaemia this varied considerably from time to time. No correlation could be demonstrated with the level of serum B_{12} and the addition of this vitamin *in vitro* did not influence the level of uptake. Uptake was greatest at 37° C and did not occur in the presence of formalin. The amount of ^{35}S methionine incorporated into the protein of the cell was remarkably constant for the normal and leukaemic cells.

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The Mononucleosis and Leukemic Cell

II. RNA Synthesis

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In previous studies [1] ribonucleic acid (RNA) synthesis in infectious mononucleosis was shown to be increased in the peripheral atypical lymphocytes at the onset of the disease when compared to normal controls. This increased synthesis declined with a corresponding decrease in atypical lymphocytes. RNA synthesis in acute leukemia was also increased in some leukemic cells, but was variable during the illness in the blast cells of the same patient and among patients with a similar type of leukemia [2, 3]. Also aberrant cytoplasmic RNA synthesis suggested differences between the benign and the malignant disorder [4]. To study further the difference of RNA synthesis between leukemic and mononucleosis leukocytes, actinomycin D was used in this investigation.

Methods and Materials

Fifteen acute leukemics were diagnosed from clinical findings, peripheral blood and bone marrow examination. Seven had acute lymphoblastic, 4 acute monocytic, 3 acute myelocytic, and 1 acute eosinophilic leukemia. When possible serial studies were performed at onset, onset after medication, remission, relapse and during the terminal state of the disease.

Eleven patients with infectious mononucleosis were diagnosed from clinical, serological, and peripheral blood findings. All were investigated early in the disease process when the percentage of atypical lymphocytes was greatest. Twelve normal individuals were used as controls.

Twenty ml of venous blood were divided in the following manner: 4 ml of blood were placed into 3 sterile plastic tubes, each containing 0.5 ml of 10% dextran in 5% glucose, and

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0.5 ml of 1% EDTA in 1.4% saline. One tube contained 5 µC of tritiated uridine (specific activity 5.0 C/mM) and was incubated for 1 h at 37°C. The remaining four tubes were incubated for 1 and 2 h with and without actinomycin D* before tritiated uridine was added. The final concentration of the drug was 5 µg/ml. After 1 h incubation with tritiated uridine, the leukocytes in the supernatant were prepared for radioassay as previously described [3]. The results were expressed as disintegrations per minute per 10^8 leukocytes. After logarithmic transformation, the differences between the samples, unexposed and exposed to actinomycin D, were analyzed by means of Duncan's method for all possible comparisons of means [5]. Since the leukemic leukocytes showed no significant difference among stages in depression of uridine incorporation by actinomycin D, they were combined into one large group for analyses.

Prior to preparation for liquid scintillation counting, autoradiographic studies were performed on the leukocytes incubated for 2 h with and without actinomycin D according to modified technique of Bond and co-workers [6, 7]. Five hundred mononuclear cells were counted and the percentage of labeled cells was determined. Background counts were evaluated and only cells containing greater than 4 grains were considered labeled. For each patient, the ratio of percentage labelling with actinomycin D to percentage labelling without actinomycin D was calculated. For each group, the geometric mean of these ratios in percentage form was calculated as measure of central tendency for the group.

Results

Clinical information. In patients with infectious mononucleosis the atypical lymphocytes ranged from 24–88%.

The white blood counts of the leukemic patients were in a comparable range to the infectious mononucleosis group. Also, in most instances, the number of blast cells did not account for the majority of cells observed. Not all patients were studied in each stage because of the nature of the disease.

Liquid scintillation. Sixty three liquid scintillation studies were performed on 12 normals, 11 mononucleosis and 15 leukemic patients. In normal subjects the proportion of disintegrations with actinomycin D at 2 h was 11% of that without the drug (geometric mean). In infectious mononucleosis patients this value was only 2%. In acute leukemic patients the result was 20%. As a measure of subject to subject variation within groups, the average coefficient of variation was 42% of these geometric mean values.

In figure 1 the ratio of results for the actinomycin treated to those for untreated leukocytes is depicted for normals, infectious mononucleosis and leukemic leukocytes. The depression of uridine uptake by the drug into leukemic leukocytes at 1 h was significantly less than either normal or infectious mononucleosis leukocytes ($P < 0.05$ and $P < 0.001$ respectively). After 2 h of inhibition with actinomycin D

* Obtained from Merck Sharp and Dohme Research Lab., West Point, Pa.

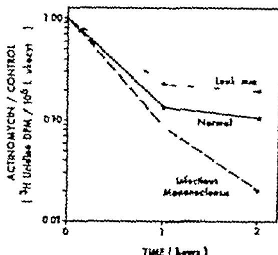


Fig 1 Ratio of results for actinomycin treated leukocytes to untreated leukocytes for the three groups.

the leukemic leukocytes continued to show less depression than those of mononucleosis (P <0.001) However no significant difference in depression was noted after 2 h exposure to the drug between normal and leukemic leukocytes.

Overall evaluation of the entire experiment revealed differences in depression of tritiated uridine incorporation between leukemic and mononucleosis leukocytes, and between leukemic and normal leukocytes (P <0.001 and P <0.05 respectively) There was also a greater depression in mononucleosis leukocytes when compared to normal white cells (P <0.001)

Autoradiography Fifty-seven autoradiographic studies were performed on 10 normals, 9 mononucleosis and 13 leukemic patients. The average grains in the mononucleosis and leukemic leukocytes were much greater than normal controls. The ratio of cells labeled with and without drug exposure at the two hour period was expressed as a geometric mean and was significant for normal (P <0.01) mononucleosis (P <0.01) and leukemic groups (P <0.05) The predominant cell labeled for mononucleosis patients was the atypical lymphocyte. In leukemics the blast cell in general showed heavier labeling but was not the predominant cell labeled. In the majority of stages (onset, onset after medication and relapse) the reduction in cell labeling was less for blasts than non-blasts.

Discussion

Actinomycin D has been demonstrated to inhibit selectively cellular DNA directed synthesis of RNA by attaching to the guanine residues [8, 9]. The drug also inhibits RNA synthesis of most DNA viruses, but permits normal or supranormal growth of most RNA viruses [8].

The marked decline in uridine incorporation, noted after mononucleosis cells were exposed to the drug, needs explanation. It would appear from the abundant number of mononucleosis cells and autoradiographic data that the drug effect was greatest in the atypical lymphocyte. Virtually nothing concerning DNA structure of mononucleosis leukocytes or drug permeability of such cells is known to explain this result. However NIEDERMAN *et al.* [10] have recently shown that a herpes-like virus may be the etiologic agent of this disease. Such herpes-like viruses have been observed in cultured cells derived from patients with infectious mononucleosis [11]. Similar viruses obtained from Burkitt lymphoma cell lines have been shown to contain DNA [12]. Also REINAUER [13] has observed particles having the morphology of viruses in the cytoplasm of a few mononucleosis cells. The presence of viral activity on a physiologic basis was further suggested by the autoradiographic results of SCHUMACHER *et al.* [14] who, using tritiated thymidine, demonstrated aberrant grains of possible viral origin in a few mononucleosis cells.

In leukemia, due to small sample size, we were unable to demonstrate statistically that the resistance was of blast cell origin. However in the majority of stages of leukemia, the blast cell showed the greatest resistance to the drug. This is supported by the findings of STORTI and TORELLI [15] who showed that RNA synthesis in leukemic blasts was less inhibited by actinomycin D than in normal myeloblasts or myelocytes. This finding cannot be explained on the basis of DNA structure [16, 17] nor can it be explained on the basis of drug action unrelated to RNA synthesis [18].

Variations in permeability of the drug could account for the result. KESSEL [19] demonstrated no difference in penetrance of the drug in normal and leukemic leukocytes, but retention was less in resistant leukemic cells. This might explain our results, but needs clarification by further experimentation on drug binding.

Since the growth of most DNA viruses is suppressed by actinomycin D [8], our findings cannot be explained by such an organism. However, uridine incorporation by an RNA virus would not ordinarily be

inhibited by the antibiotic [8] At the present time four viruses have been associated with human leukemia [20] One is the herpes-like virus similar to the one previously described in mononucleosis. The other three belong to groups which are known RNA viruses [21] If leukemia is a viral disease, viruses of these latter groups would best explain the findings in this investigation.

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Summary

In vitro studies employing autoradiographic and liquid scintillation techniques on leukocytes from 12 normal individuals, 11 patients with infectious mononucleosis, and 15 patients with acute leukemia were performed. The incorporation of tritiated uridine into the peripheral blood leukocytes of these groups was studied with and without exposure to actinomycin D. As shown by quantitative results, the incorporation of tritiated uridine into mononucleosis leukocytes was depressed more by actinomycin D than were the other groups. Autoradiographic results showed that the mononucleosis cells were those markedly depressed by the drug The incorporation of tritiated uridine into leukemic leukocytes was depressed less by the drug than were the other groups. The leukemic blast cell may have been responsible for the quantitative abnormality

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Modes of Presentation of *Thalassaemia minor*

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This short paper describes the various clinical pictures under which patients with thalassaemia minor came to seek medical advice. Patients with the anomaly known prior to their first visit have not been included. The present material consists of 260 patients seen in my wards and my private practice during the last ten years. Mainly adults are included, as children are usually referred to pediatric units or to consultant pediatricians. There were 140 males and 120 females; their age varied from 16 to 62 years. The laboratory criteria for establishing the diagnosis of thalassaemia minor were: Morphological changes of the erythrocytes, increase of HbA_2 above 3.8%, increased serum iron and frequently presence of HbF.

1. Anaemia. 123 patients came for anaemia of unknown origin, the Hb-level varying from 7.5 to 11.5 g/100 ml. Many of these patients were previously treated for iron deficiency anaemia, naturally without success: it was for this refractoriness to iron treatment that they were referred. Table I gives the distribution of the Hb levels of these patients.

Table I. Hb levels on 123 patients with thalassaemia minor

g Hb/100 ml	Number of patients
7–8	6
8–9	16
9–10	25
10–11	52
> 11	24

2 Splenomegaly 14 patients were referred on account of a splenomegaly found by their doctor on a routine examination.

3 Hepatomegaly In 32 patients the presenting finding was hepatomegaly of various degree Flocculation tests were occasionally abnormal, but the transaminase levels were within normal limits.

4 Episodes of mild jaundice 16 patients came with a history of repeated attacks of mild jaundice. Some of them were considered as suffering from mild chronic hepatitis, whilst some others came with a diagnosis of congenital hyperbilirubinaemia.

5 Loss of energy and lassitude 12 patients complained of fatigue and lassitude. As the ordinary blood test had shown no significant anaemia, the complaints were attributed to psychoneurosis. There was no correlation between the Hb-level and the intensity of the symptoms. The lack of energy could perhaps be attributed to impaired synthesis of the various intracellular iron-containing enzymes (cytochrome, peroxydase, etc.) Information regarding the disturbance of these enzymes in thalassaemic syndromes is very scanty

6 Gall stones. 22 patients came on account of gall stones. The young age of most of these patients was suggestive of a chronic haemolytic condition.

7 Mild pyrexia. 5 patients were referred for mild pyrexia (98.6–99.2 F) for which no cause was found in spite of a complete laboratory and radiological investigation Most patients were young There is no plausible explanation for this mild pyrexia in thalassaemia minor

8. Erythrocytosis 4 patients were referred for erythrocytosis with RBC counts varying from 6 to 6.5 millions/mm Two of them were thought of suffering from polycythaemia vera. Naturally all these four patients had an Hb level below normal and the mean corpuscular haemoglobin was characteristically low Erythrocytosis in a few patients with thalassaemia minor is a known fact.

9 Leucocytosis 4 patients were referred because their white cell count was constantly raised, varying between 10,000 and 13 000/mm These patients had already undergone a thorough laboratory investigation for detection of the cause of the leucocytosis, but this had remained obscure. It seems probable that the raised activity of the bone marrow brought about by the increased rate of haemolysis was the cause of the leucocytosis

10 Premature beats 2 persons aged 32 and 45 respectively had been suffering from extrasystoles. Complete physical examination was negative, and the ECG apart from ventricular premature beats

Table II The presenting disturbance which led to the diagnosis of thalassaemia minor in 260 cases

Symptoms or signs	Number of Patients	%
Anaemia	123	47
Splenomegaly	14	5.4
Hepatomegaly	32	12.3
Jaundice	16	6.2
Loss of energy and lassitude	12	4.6
Gall stones	22	8.5
Mild pyrexia	5	1.9
Erythrocytosis	4	1.5
Leucocytosis	4	1.5
Extrasystoles	2	0.8
Accidental findings	26	10

showed no abnormality One might surmise that a certain degree of haemochromatosis of the heart is the cause of this disturbance. The heart failure which often is the cause of death in thalassaemia major is attributed to haemochromatosis of the cardiac muscle, the excessive erythropoiesis in the overactive marrow itself stimulating the bowel to over-absorption of iron which causes its deposition in the tissues [LEHMANN]

11 Finally there were 26 cases who came with various complaints, the detection of the thalassaemic trait being completely accidental.

Table II summarises the various disturbances which led to the diagnosis of thalassaemia minor

Summary

The clinical modes of presentation of thalassaemia minor in 260 Greek adult patients are given.

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Reticulocyte and Normoblast Labelling with Leucine – H in Thalassaemic and non Thalassaemic Subjects

M. Kesse Elias, E. Gyftaki, V Alevizou Terzaki and A Koukoulommati

The purpose of the present work is to study by autoradiography in thalassaemic immature red cells, appearing in the peripheral blood, the capacity to synthesize haemoglobin and compare the data with those of non thalassaemic subjects. In the peripheral blood of thalassaemic patients the presence of nucleated red cells, in addition to a high number of reticulocytes, is a characteristic finding [9] Non thalassaemic patients with immature forms of the red series in their peripheral blood were selected for comparison.

During the course of maturation erythroid cells lose the ability to synthesize DNA and to divide and their nucleus is lost [5] It is known further that in reticulocytes after the loss of the nucleus, protein synthesis continues, and that the synthesized protein is haemoglobin The three types of RNA known to play a role in protein synthesis must be present in reticulocytes. Since no synthesis of new RNA occurs in reticulocytes, RNA synthesized in the nucleus and migrating to the cytoplasm is retained in the cytoplasm after the loss of the nucleus [6] Any reticulocyte, coming from a normoblast, has therefore a supply of RNA in its cytoplasm.

The basophilic network, which appears in the reticulocytes after supravital staining contains RNA [3] and it is likely that throughout the life of the reticulocyte there is a gradual decrease in the amount of RNA containing reticulum with a slowing down of protein synthesis [6]

The present study has been performed *in vitro* In order to follow haemoglobin synthesis, peripheral blood was incubated with leucine – H and the smears were autoradiographed

Table I. Haematological data (continued)

Case No.	Age years	Sex	Haematological status	Hb g%	RTC %	Nucleated red cells /100 white cells	Erythroblasts with traces of basophilia %	Orthochromatic erythroblasts, %	Hb electrophoresis
15	30	F	Thalassemia major splenectomy	6.8	3.1	4.0	0	100.0	A A_2 F 40%
16	16	F	Thalassemia major splenectomy	6.8	11.4	376.0	22.8	97.2	A, A_2 F 80%
17	9	F	Thalassemia major splenectomy	7.4	8.4	994.0	22.3	77.7	A>F A_2
18	7	M	Thalassemia major splenectomy	8.4	6.5	250.0	8.4	91.6	A_2 F>A
19	10	F	Thalassemia major splenectomy	9.0	3.0	20.0	22.5	77.5	A, A_2 F 60%
20	27	F	Sickle cell-Thalassemia, splenectomy	8.7	17.6	345.0	9.7	90.3	S≫F A
21	28	F	Spleen lymphosarcoma	8.9	1.5	20.6	100.0	0	
22	28	M	Acute leukaemia	9.3	2.1	3.4	39.0	61.0	
23	48	F	Posthaemorrhagic anaemia	8.0	1.3	1.0	66.6	33.3	
24	48	M	Refractory anaemia	6.0	3.6	4.0	80.5	19.5	
25	23	M	Favism	6.7	7.8	3.0	75.0	25.0	
26	19	M	Splenectomy after an accident	14.3	5.0	5.0	100.0	0	
27	24	F	Refractory anaemia	6.8	3.8	24.0	81.0	9.0	

Table II. Labelling of erythroid precursors of peripheral blood with leucine –³H

Case No.	Haematological status	Labelled RTC %	Labelled erythroblasts with traces of basophilia, %
1	Thalassaemia major	68.6	0
2	Thalassaemia major	63.6	0
3	Thalassaemia major	73.5	0
4	Thalassaemia major	64.4	0
5	Thalassaemia major	71.9	55.2
6	Thalassaemia major	58.6	68.6
7	Thalassaemia major	62.9	100.0
8	Thalassaemia major	63.2	72.5
9	Thalassaemia major	61.6	100.0
10	Thalassaemia major	64.5	100.0
11	Thalassaemia major splenectomy	86.9	67.0
12	Thalassaemia major splenectomy	56.1	100.0
13	Thalassaemia major splenectomy	62.5	77.5
14	Thalassaemia major splenectomy	53.2	92.2
15	Thalassaemia major splenectomy	69.8	0
16	Thalassaemia major splenectomy	56.9	100.0
17	Thalassaemia major splenectomy	72.4	78.9
18	Thalassaemia major splenectomy	73.5	76.1
19	Thalassaemia major splenectomy	55.2	90.6
20	Sickle cell-Thalassaemia, splenectomy	60.0	24.7
		mean 64.8	mean 80.0
21	Spleen lymphosarcoma	71.4	100.0
22	Acute leukaemia	68.2	66.6
23	Posthaemorrhagic anaemia	70.4	100.0
24	Refractory anaemia	72.0	100.0
25	Favism	64.7	90.9
26	Splenectomy after an accident	62.0	100.0
27	Refractory anaemia	67.5	89.6
		mean 67.7	mean 92.4

None of the orthochromatic erythroblasts was found labelled wit leucine –³H

Results

In thalassaemic subjects most of the erythroblasts appearing in the peripheral blood, are orthochromatic with fully acidophilic cytoplasm and pycnotic nucleus wihout any structure (table I fig 1b) None of these orthochromatic erythroblasts was labelled with leucine -^{3}H. Table II gives the data on the percentage labelling of reticulocytes and normoblasts, containing traces of basophilic substance in their cytoplasm, from thalassaemic and non thalassaemic subjects.

In the 19 thalassaemic subjects and one with S-thalassaemia, the mean value of the labelling index (= % labelled cells) is 64.8 for reticulocytes and 80 0 for erythroblasts containing in the cytoplasm basophilic substance and in non thalassaemic 67 7 and 92 4 respectively

Correlation of the labelling index between the two groups studied revealed for reticulocytes $P > 0.2$, for erythroblasts containing in their cytoplasm basophilic substance $P > 0.1$ Consequently there is no difference in the labelling index between the two groups either for reticulocytes or for erythroblasts.

Comments

Comparing the two groups studied, it is obvious that there is not any significant difference in the labelling index either for reticulocytes or erythroblasts containing in their cytoplasm traces of basophilic substance. It seems that for the same last steps of erythroid cell precursors no difference between thalassaemic and non thalassaemic subjects can be detected concerning the capacity of the cells to synthesize haemoglobin. No quantitative informations are drawn through this study

Reticulocytes seem to be more immature than fully acidophilic normoblasts. It would thus appear that reticulocytes arise from more immature erythroblasts as has been stated by other investigators after incubation of bone marrow with ^{59}Fe and autoradiographic examination of the cells [4 7]

The high percentage of completely acidophilic unlabelled normoblasts in peripheral blood of thalassaemic subjects is explained if we accept the hypothesis that the loss of the nucleus in bone marrow is slowed down in late stages of erythroid cell precursors [1] This would allow a large proportion of normoblasts to undergo numerous mitoses,

although the cytoplasm matures, filling up the bone marrow with orthochromatic erythroblasts and causes an increase of bone marrow output in orthochromatic erythroblasts [8]

Acknowledgements

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Summary

Peripheral blood from thalassaemic and non thalassaemic patients was incubated for 3 h with leucine –³H. The selected non thalassaemic patients had immature forms of the red series in their peripheral blood. Smears were prepared and autoradiographed. None of the fully orthochromatic erythroblasts was found labelled with leucine –³H. The labelling index of the two groups studied did not show any difference for reticulocytes or erythroblasts containing traces of basophilic substance in their cytoplasm.

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A Survey for Erythrocyte Glucose-6-Phosphate Dehydrogenase Deficiency in Rumania

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Hemolytic disorders following consumption of certain vegetables and drugs have long been known, but only 12 years ago it was recognized that the deficiency of erythrocyte glucose-6-phosphate dehydrogenase (G-6-PD) is in the background of these disorders [2] The G-6-PD deficiency is prevalent amongst some ethnic groups including American and Africans Negroes, Caucasians of Mediterranean countries and some South-east Asian populations [6 8 9 12] In Central and Northern Europe, the G-6-PD deficiency appears too, but only sporadically [3 4 5 13]

In Rumania, the first case of G-6-PD deficiency – a chronic non-spherocytic anemia – was observed in 1964 by Kondi *et al.* [7] One year later we reported two cases of favism and the investigation of the relatives revealed other three defective subjects [10] The absence of previous data regarding the incidence of this enzymopathy in our country prompted us to conduct a search for G-6-PD deficiency among a Rumanian population and in 1966 we exposed the preliminary results [11]

The purpose of this paper is to report the actual data of our survey

Materials and Methods

Venous blood specimens were obtained from randomly selected patients and normal individuals, from the Department of Paediatrics and the Policlinic of Husi (Rumania).

The qualitative G-6-PD activity was determined on freshly-drawn heparinized blood, by the methemoglobin reduction test of Brewer *et al.* [1], Subsequently the deficient individuals and their relatives were investigated clinically hematologically and genetically

Table. G-6-PD deficient individuals

No.	Subject	Years	Sex	Pedigree	Diagnosis	Methemoglobin reduction test
1	G. O	3	M	propositus	favism	complete deficiency
2	G. R.	25	F	mother	healthy	partial deficiency
3	C. V	28	F	aunt	healthy	partial deficiency
4	C. A.	2	M	cousin	healthy	complete deficiency
5	M. D	1	M	propositus	favism	complete deficiency
6	M. C.	29	F	mother	healthy	partial deficiency
7	B. C.	49	F	grandmother	healthy	complete deficiency
8	B. V	11	M	uncle	healthy	complete deficiency
9	G. C.	15	M	propositus	hemophilia A, acut hemolytic anemia	complete deficiency
10	G. L	37	F	mother	healthy	complete deficiency
11	S. V	17	M	propositus	healthy	complete deficiency
12	S. E.	47	F	mother	healthy	partial deficiency
13	S. R.	9	M	brother	healthy	complete deficiency
14	S. F	7	M	brother	healthy	complete deficiency
15	D. A.	64	F	grandmother	healthy	complete deficiency
16	B. E.	37	F	aunt	healthy	partial deficiency
17	A. A.	0.5	F	proposita	acute hemolytic anemia	complete deficiency
18	A. L.	43	M	father	healthy	complete deficiency
19	L. G.	66	M	propositus	thalassaemia minor	complete deficiency
20	H. M.	27	F	daughter	healthy	complete deficiency
21	D. A.	31	F	daughter	healthy	complete deficiency
22	D. V	4	M	grandson	adactylia of the right hand	complete deficiency
23	G. A.	31	F	proposita	history of favism	complete deficiency
24	G. E.	73	F	mother	chronic anemia	complete deficiency
25	G. D.	81	M	father	healthy	complete deficiency
26	G. E.	13	F	daughter	healthy	partial deficiency
27	G. C.	9	M	son	healthy	complete deficiency
28	O G.	3	M	propositus	favism	complete deficiency
29	O A.	24	F	mother	healthy	complete deficiency
30	D. C.	68	M	grandfather	healthy	complete deficiency
31	M. T	35	F	aunt	healthy	complete deficiency
32	M. P	6	M	cousin	healthy	complete deficiency

In this study the screening test employed, did not detect as propositus, partially G-6-PD deficient females.

Results

The methemoglobin reduction test was performed on 890 males and 792 females. Complete G-6-PD deficiency was found in 8 probands

and the survey of 94 relatives revealed other 24 deficient individuals. All the deficient subjects are of Rumanian origin between their families are no relation. The clinical and hematological data for these 32 deficient individuals are listed in the table

Discussions

This is the first attempt at estimating the incidence of G-6-PD deficiency in a Rumanian population, randomly selected. Considering only the test performed on males, the G-6-PD deficiency appears to attain 1.8% (gene frequency 0.0067–0.018)

It is difficult to explain the appearence of the enzymopathy Since in our country malaria was endemic in previous years, the G-6-PD deficiency could be explained by the controversial malaria theory Another possibility is that the genopathy was brought by the mediterranean peoples who colonized the ancient territory of Rumania (the ancient Dacia)

We suppose that the G-6-PD deficiency is not restricted to the area investigated in our survey and we think that it would be advisable to continue such studies, not only for the genetic and anthropological significance of this estimation but also for the prevention of the hemolytic states in defective subjects.

Summary

A survey for erythrocyte glucose-6-phosphate dehydrogenase deficiency was carried out amongst 1682, randomly selected Rumanian individuals, by the methemoglobin reduction test. 32 individuals were found to have a G-6-PD deficiency Considering only the G-6-PD deficient males, the enzymopathy appears to attain 1.8% (gene frequency: 0.0067–0.018).

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Acid Phosphatases of Platelets during Coagulation and Storage of Blood

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Platelet factor 3 (PF3) belongs to the basic properties of platelets by which the haemostatic function of these blood elements is realized. It is evident that we can speak about lipoprotein the exact localization of which in the ultrastructure of platelets is still open [1].

In the previous work it was shown that the acid phosphatases (APh) liberated from the platelets into serum during the coagulation of blood are probably linked to lipoprotein which is responsible for the so called platelet like activity of serum [2].

In this work we tried to find the basic correlation between activity of APh and PF3. From the point of view of lysosomal theory APh can be used as biochemical marker of lipoprotein of lysosomal origin [3].

Material and Methods

Blood was taken from healthy volunteers. The serum for studying PF3 like activity was acquired from native platelet rich plasma (PRP) without use of anticoagulant as described previously as well as the setting of the activity of APh of serum or plasma without the use and with the use of detergent-analogon Triton X-100 [2].

The shortest fibrinogen time (SFT) was set as O'Brien described [4].

APS ("activité plaquettaire du sérum") was carried out according to Alagille and Soulier [5]. Results are given as differences in prothrombin consumption between 4-6 min after recalcification of test mixture. The substrate plasma without platelets was prepared for both coagulation tests according to the last mentioned method.

The fresh blood stored in ACD solution was kindly supplied by Transfusion Service Žilina. The blood was stored at 4°C and in certain intervals the samples were taken under sterile conditions. Platelets were counted by the Feissly method [6]. Plasma was separated from the samples by centrifugation at 4°C at 2,000×g twice each centrifugation lasting

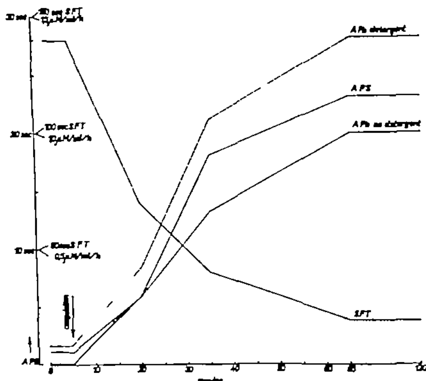


Fig. 1 Time dependence of liberation of acid phosphatase (APh) and PF3 like activity into serum from native platelet rich plasma (PRP). *SFT* = shortest fibrinogen time *APS* = platelet activity of serum.

30 min. No formed elements could be observed in this plasma under the phase microscope. In the plasma we observed the haemoglobin content [7], the activity of APh without and with detergent.

The thromboplastic activity by means of methods mentioned above [4 5] with modification forced by using of citrate plasma instead of serum as tested material. The basic mixture which was the same for both methods contained 0.3 ml of platelet poor substrate plasma and 0.2 ml of tested plasma. T recalcification 0.5 ml of 0.025 M $CaCl_2$ were used.

Haemolysate was prepared by dilution of isolated erythrocytes by destilled water to desired concentration of haemoglobin [8].

Results

The time dependence of liberation of APh and PF3 activities into serum is given in figure 1 The curves show the correlation between both activities. The activities in serum seem to depend on the retrac

Table II. Changes in some activities in plasma from blood stored in ACD solution at 4°C

Blood No.	Day	Thrombocytes	Haemoglobin mg%	Acid phosphatase μM/ml/h no deterg.	with deterg.	SFT sec	APP sec
	0	163 000	10	0.140	0.216	68	0
1	7	152 000	20	0.224	0.714	24	3.7
	14	94 000	66	0.478	0.840	16	12.3
	21	50 000	208	1.204	2.534	15	20
	0	223 000	14	0.250	0.420	52	2
2	7	192 000	14	0.345	0.870	30	8
	14	160 000	47	0.435	0.920	22	10
	21	101 000	71	0.770	1.540	20	20
	0	217 000	28	0.226	0.332	31	0.5
3	7	168 000	48	0.546	1.172	18	18
	14	130 000	126	0.868	1.680	18	23
	21	66 000	181	0.742	1.594	18	23
	0	186 000	16	0.064	0.112	110	0
4	7	150 000	50	0.350	0.560	31	5
	14	101 000	128	0.882	1.358	20	20.2
	21	93 000	198	0.700	0.994	18	14.2
	0	207 000	28	0.126	0.196	66	0
5	7	128 000	48	0.294	1.134	17	10.5
	14	98 000	144	0.588	1.280	17	16.3
	21	48 000	226	1.148	2.072	17	22

HOROWITZ *et al.* [9] shows as for quantitative changes after ultracentrifugation the similar values as the activity of APh. About 15–20% of the activity APh liberated into serum remains also after centrifugation at 20 000×*g* in the supernatant. In such supernatant no tromboplastic activity could be traced by coagulation tests we used.

When following the curves showing the changes of given values in plasma obtained from the stored blood, the time correlation of the appearance of thromboplastic activity concomitantly with the activity of APh is also evident. We assume that we dealed with material of platelet origin according to WOLF [10] who described the platelet dust liberated into plasma during storage of blood. The enormously high values of APh obtained especially by means of detergent indicate that there must be a rest of the platelet lysosomes. This should prove electronoptical findings [11]. Even if the older samples of plasma show comparatively high values of haemoglobin, erythrocytes participated in both observed activities only very little. Haemolysate of isolated

erythrocytes the value of which was 200 mg% haemoglobin shows the activity of APh with detergent about 0 1 units of Bessey Lowry No thromboplastic activity could be traced by tests used by us.

The fact that during storage of blood, except others, also the rest of platelet lysosomes is liberated into plasma is interesting both from theoretical and from practical points of view If the correlation between by APh labeled lipoprotein and PF3 activity is proved by further experiments, it could help to solve the question of PF3 origin. We should also mention the finding of Kowalski *et al.* [12] that platelet APh is activated by kaolin when the values approached the values obtained by means of Triton X 100 only On the other side the activity of PF3 was proved by means of the very kaolin [13 14]

The fact that highly active sediment of serum acquired by ultracentrifugation is completely captured in coagulum of native PPP might mean that during retraction of coagulum a part of thromboplastic activity liberates into serum and perhaps is to ensure the perfect course of coagulation.

The increased availability of PF3 in plasma of stored blood could probably help to explain the well known clinical experience regarding the unsuitability of transfusion of stored blood to patients suffering from thromboembolic diseases.

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Summary

A time correlation of appearance of acid phosphatase and platelet factor 3 activities in the serum obtained from native platelet rich plasma has been found. But none of these activities was liberated from the platelets into plasma or serum close to the time when the coagulum developed *in vitro*. The liberation of both activities could be observed only after retraction of the coagulum.

Concomitantly with the liberation of acid phosphatases the platelet factor 3 activity in plasma obtained from stored blood raised. The high values of acid phosphatases in plasma give evidence to their platelet lysosomal origin.

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Bone Marrow Injury and Repair

Irradiation and Mechanical Disruption[1]

M. B. Weinstein[2] and W. H. Crosby

Exposure of the femur and tibia of the rat to 4 000 r of x irradiation results in permanent aplasia of the bone marrow [1]. Two months post irradiation, when the aplasia has been well established, autologous marrow removed from an unirradiated bone and implanted in the irradiated area can reconstitute hemopoietic activity [2]. It has been proposed that the limiting factor which determines the existence of aplasia or hemopoiesis is the condition of the microcirculation of the marrow. This sinusoidal structure is evidently uniquely capable of supporting the proliferation of hemopoietic tissue. When it is destroyed by irradiation, or otherwise, the hemopoietic marrow atrophies. The restoration of normal activity in the irradiated marrow cavity by transplantation of tissue is presumably accomplished by adventitial cells capable of proliferating to reconstitute the microcirculation. This experiment was designed to learn if normal marrow from the shielded portion of an irradiated bone could be induced by mechanical mixing to proliferate in the aplastic portion.

Materials and Methods

Radiation was performed on the distal half of the tibia in groups of 10 male Wistar rats of about 400 g. The rats were placed in individual boxes and shielded by layers of lead to protect the body, upper legs and feet. The radiation source was 250 kvp-15 mA with ½ mm

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[2] Trainee in Hematology supported by US Public Health Service Grant No. AM 5210-07

copper and 1 mm aluminium filter The target-to-skin distance was 35 cm. Dose rate was 250 r/min, and exposure time was 13.5 min. The half-value layer was 1.2 mm copper The total exposure dose was 4,000 r The original groups of 10 rats were reduced by 20 % because of autoamputation of irradiated legs. Shielding the feet during irradiation prevented this.

When anesthesia was required, pentobarbital was injected intraperitoneally Mechanical disruption of tibial marrow was accomplished by introducing a 1½ in, 23 gauge needle into the marrow cavity at the femorotibial joint. Then a stainless steel wire was passed through the needle into the cavity and the marrow was mixed by 20 up-and-down strokes of the wire trochar

The marrow was disrupted t varying intervals after the irradiation (table I). In one group of animals the operation was performed before irradiation and in one group it was performed without irradiation. Only marrow of the left leg was disrupted so that the right could serve as control.

The animals were killed with ether and the tibias were removed and fixed in buffered formalin for 3 days, decalcified in Perenyi's solution for 5 days, imbedded in paraffin, sectioned at 6 μm and stained with hematoxylin and eosin.

Results

Those bones in which the marrow was not mechanically disrupted were examined at intervals up to 8 weeks after irradiation and the typical cycle of changes was observed sinusoidal dilation, intra medullary hemorrhages, hyperplasia and then hypoplasia [1] In all

Table I. Recovery of hemopoiesis in hemiirradiated tibia 4000 to distal end followed by mechanical disruption of marrow

Group (10 rats each)		Marrow disrupted after irradiation weeks	Interval from disruption of marrow until death, weeks	Interval from irradiation of marrow until death, weeks	Recovery of hemopoiesis
1	A	1	9	10	yes
	B	1	13	14	yes
	C	1	15	16	yes
2	A	2	6	8	yes
	B	2	8	10	yes
3	A	4	4	8	yes
	B	4	6	10	yes
	C	4	8	12	no
	D	4	10	14	no
4	A	6	4	10	no
	B	6	6	12	no
	C	6	10	16	no
5	A	8	2	10	no
	B	8	6	14	no

of the irradiated tibias there was a line of demarcation by the 8th week in the distal end of the cavity the hemopoietic marrow was replaced by loose connective tissue, rare lymphocytes and plasma cells and few erythrocytes. The proximal end contained normal marrow

The effect of mechanical disruption of marrow in the unirradiated tibia was studied after the 5th postoperative day Marked endosteal proliferation was seen and in some areas the medullary cavity became completely filled with trabecular bone. Two, 3 and 4 weeks after disruption of the marrow some trabecular bone remained in the cavity but endosteal proliferation subsided with time.

Disruption of marrow preceding irradiation by 5 days did not modify the effects of irradiation. At 8 weeks there was fibrosis in the distal end of the cavity and in the proximal end there was normal hemopoietic marrow plus trabecular bone. The irradiated distal right tibia in every animal became fibrotic and aplastic. Results of the combined injury are shown in table I. When mechanical disruption of the left tibial marrow followed irradiation by one or two weeks (groups 1 and 2) hemopoietic cells were found in the irradiated areas 4 to 15 weeks postoperatively Hemopoietic tissue and trabecular bone were present through the entire length of the cavity In some bones islands of normal tissue were scattered among aplastic areas. When disruption followed irradiation by four weeks (group 3) there was recovery of hemopoiesis in the distal cavity of some animals, but not all. When disruption followed irradiation by 6 or 8 weeks (groups 4 and 5) there was only fatty and connective tissue in the distal end of the cavity 2 to 10 weeks postoperatively

Discussion

When the marrow was mechanically disrupted, the cavity quickly became filled with trabecular bone surrounded by osteoblasts. The endosteum was hypertrophic. In succeeding weeks the hypertrophy subsided. The bony replacement was complete in some bones, patchy in others. Hemopoietic marrow was present in the areas of no bone and in the spaces between the bony trabeculae.

When the marrow was mechanically disrupted after irradiation, the irradiated and unirradiated tissues were tumbled and mixed within the bony cavity The spotty recovery of the marrow in the irradiated portions of the bone indicate that the intent of the procedure was

accomplished. Normal marrow was forced into the aplastic tissue and established itself there. But the presence of aplastic areas in the proximal cavity suggests that irradiated tissue was moved into the shielded areas and it was not invaded or converted by the adjacent normal tissue.

As the irradiation lesion matured it became more fibrotic. After four weeks the efforts to mix the marrow in the two ends of the cavity were unsuccessful, perhaps because the wire trochar only channeled the irradiated tissue and did not replace it.

It is proposed that the growth of hemopoietic marrow in irradiated portions of the tibia occurred because adventitial tissue was displaced into those areas permitting the marrow s microcirculation to be reestablished there. This would indicate that the marrow s adventitia is not aggressive it does not propagate into irradiated areas by its own initiative. Hemopoietic tissue on the other hand, seems to develop wherever there is adventitia to support it.

Summary

Post-irradiation aplasia in the marrow of the rat's tibia developed only in the irradiated distal half. When the marrow of the unirradiated half was mixed into the irradiated portion by means of mechanical disruption of the bony cavity hemopoietic marrow developed in the irradiated portion.

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Eosinophils and Dietary Histamine in the Rat

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Eosinophils are most numerous in the gastrointestinal tract and the skin and spend most of their life in these tissues [1]. Their function is still obscure, but Archer [2] considered that they inactivated histamine: this conclusion was based on the occurrence of a local eosinophilia following intradermal injection of histamine, which could be inhibited by injection of an antihistamine immediately after the injection of histamine. This hypothesis would be strengthened if the turnover or number of eosinophils altered in response to an alteration in histamine balance or in response to the continuous administration of antihistamine. In the present work, continuous infusions of tritiated thymidine were used in rats to investigate the effect of histamine-high diet, histamine-low diet and normal diet with antihistamine, on eosinophil turnover and populations. One of the authors has previously studied eosinophils in normal rats by this technique [1].

Material and Methods

Seven (B 1–7) adult male black-hooded PVG/C rats (weight 300 g) and 16 (S 1–16) adult male Sprague-Dawley albino rats (weight 180–210 g) were divided into 4 groups according to diet. Each diet was given for 16 days before the rats were killed. In each group, 2, 3 or 4 rats were given 8 or 6 days continuous intraperitoneal infusion [3] of 1 μc tritiated thymidine (specific activity 3.0 C/mmol)/g/day, starting 8 or 10 days after the special diets were begun and ending when the animals were killed. The detailed treatments in each group were as follows.

Normal diet. Six control rats (B 1–2 and S 1–4) received a normal cubed 41 B diet [4]. Three were infused with tritiated thymidine for 6 days (B 1 and S 1–2) and one for 8 days (B 2).

Histamine-high diet. Seven rats (B 3–5 and S 5–8) each received 15 g fresh minced raw meat daily. This diet supplies large quantities of exogenous histamine and probably also stimulates the formation of endogenous histamine [5]. Three rats (B 3 and S 5–6) were infused with tritiated thymidine for 6 days.

Histamine-low diet. Six rats (B 6–7 and S 9–12) each received 15 g daily of diet containing less than 0.05 μg histamine/g [6]. One rat was infused with tritiated thymidine for 8 days (B 6) and 2 for 6 days (S 9–10).

Antihistamine. Four rats (S 13–16) on a normal 41 B diet each received 2.5 mg Mepyramine maleate/day. This was given in the drinking water to S 15–16 throughout, and in the drinking water to S 13–14 for 10 days, then in a continuous intraperitoneal infusion for 6 days. Rats S 13–14 were infused with tritiated thymidine for 6 days.

After 10 days of the special diets, absolute eosinophil counts [7] were made on blood samples taken from 4 rats in each group (S 1–16). Blood films were taken from the tail veins of each infused rat at intervals during the infusion and fixed in 100% methyl alcohol, marrow smears (fixed in 100% methyl alcohol) and tissue blocks (fixed in formol-saline) were taken at necropsy from each infused rat. Tissue blocks were also taken at necropsy from rats B 4–5 (histamine-high diet) and B 7 (histamine-low diet). Sections (5 μm) were prepared from the tissue blocks; these from the infused rats, and their blood films and marrow smears, were dipped in Ilford K.5 nuclear emulsion or covered with Kodak AR 10 stripping film and these autoradiographs were exposed for 4 weeks at 0–4 C. All the sections, films and smears were stained with Mayer's haemalum and Biebrich scarlet to identify the eosinophils [8]. Labelled and unlabelled eosinophil nuclei to a total of between 50 and 200 were counted in each film, smear or section. Eosinophils in the marrow were not differentiated into myelocyte, metamyelocyte, stab form or mature eosinophil.

Results

The marrow smears from each infused rat showed that cells with eosinophil granules formed 8–10% of the total cell population, and the percentage labelling of these cells was 97–100%.

In the black hooded rats, differential cell counts on the blood films showed that eosinophils formed 3% of the circulating white cells, and between 100 and 120 eosinophils were present in each film. These values are within normal limits [1]. In the Sprague-Dawley rats, absolute eosinophil counts on blood samples were between 122 and 288 eosinophils/mm^3 which is within the range given by RYTÖMAA [9] for normal rats weighing 210 g. Thus the total number of blood eosinophils was within the normal range irrespective of diet.

The percentage of eosinophils labelled in each blood film was used to establish their rate of turnover. Sequential entry to and random removal from blood has been established for normal rat eosinophils [1] and this requires that

$$\log\left(\frac{1}{1-f}\right) = kt,$$

where f is the fraction of eosinophils labelled at time t, and k is the number of eosinophils added to the circulation in unit time, expressed as a fraction of the number normally present in the circulation. If log $(1/1-f)$ is plotted against t and a best fitting line is drawn, the slope of this line gives the value of k. k was determined in this way for each rat, and $(1/2k)$ (the half life of an eosinophil in the circulation) is given in table I. The intercept of the line on the abscissa gives the time at which the eosinophils begin to leave the bone marrow and enter the blood. The time between the start of the infusion and this point is the length of time from the end of deoxyribonucleic acid synthesis of an eosinophil myelocyte to the exit from the marrow of the consequent eosinophil leucocyte (table I). The half life of eosinophils in the blood, and the time of appearance of labelled eosinophils in the blood, do not differ significantly from the normal in any dietary group.

In the tissues the distribution of eosinophils was normal in all rats [9]. No significant difference was found in either total number or percentage labelling of eosinophils between black-hooded and Sprague Dawley rats, and the values for the two strains of rat have therefore been pooled for the statistical analysis; there was also no significant difference between the percentage labelling of eosinophils in stomach, small intestine and large intestine, which have therefore also been

Table I. Half-life of eosinophils in the blood and interval between beginning of tritiated thymidine infusion and appearance of labelled eosinophils in the blood

Rat	Diet	Half-life h	Time of appearance of labelled eosinophils h
B 1	Normal	8–12	60–73
B 2			
B 3	Histamine-high	11.5	67½
B 6	Histamine-low	10.1	61
S 1	Normal	10.0	51
S 2		8.8	50
S 5	Histamine-high	8.4	40
S 6		10.0	40
S 9	Histamine-low	5.9	44
S 10		8.0	48
S 13	Normal diet with antihistamine	9.8	46
S 14		8.3	42

Range of normal from Foot [1].

pooled for the analysis. The mean values and the standard deviations for total number and percentage labelling of eosinophils in the tissues are given in tables II and III and comparison using the Student t test showed no significant differences between the dietary groups.

Discussion

CODE [10] suggested that eosinophils contained histamine, but the amount is insignificant when compared to that in mast cells [11].

There is good evidence that the eosinophil is an antagonist to histamine. This property has been demonstrated in eosinophils from rabbits [12], from horses [13, 14], and from man [14]. WELSH and

Table II. Total number of eosinophils in 2 mm^2 of a 5 μm section from each rat tissue: mean and standard deviation for each dietary group

Diet	No. of rats	Spleen	Stomach	Small intestine	Large intestine
Normal	4	23± 4	208±68	130±35	145±52
Histamine-high	5	19± 3	233±75	132±36	139±21
Histamine-low	4	23±12	204±64	132±24	167±34
Normal diet with antihistamine	2	19± 6	164±53	111±38	177±40

Table III. Percentage of eosinophils labelled in rat tissues: mean and standard deviation for each dietary group

Diet	No. of rats	Spleen	Stomach and small and large intestine
	6-day tritiated thymidine infusion		
Normal	3	79±10	43±21
Histamine-high	3	70± 4	40±14
Histamine-low	2	70± 7	37±16
Normal diet with antihistamine	2	74± 9	50±19
	8-day tritiated thymidine infusion		
Normal	1	88	79± 8
Histamine-low	1	83	83±10

GREER [15] produced electron micrographs which demonstrated phagocytosis of mast cell granules by eosinophils in the peritoneal fluid of a rat.

There is also good evidence of a chemotactic effect of histamine on eosinophils in some species but not in others. When lesions of urticaria pigmentosa were stroked they showed some degranulation of the mast cells with a slight influx of eosinophils [16] and in man marked local eosinophilia followed injection of histamine in the skin of atopic individuals, although this did not occur in those who were not atopic [17 18] Marked local eosinophilia followed injection of histamine in horses [2 19] but this effect was not seen in mice [20 21] guinea pigs [22] or dogs [23]

Mast cell granules contain most of the histamine in the body [24] and these granules may be released in the immediate hypersensitivity reaction [25] or in acute inflammation [26] thus producing a local eosinophilia. SHELDON and BAUER [26] showed that damage to the skin of rats produced by experimental mucormycosis caused degranulation of local mast cells within half an hour followed by a local eosinophilia in about 6 h pretreatment with compound 48/80 (a histamine liberator) caused degranulation of the mast cells, and inflammation was then not associated with a local eosinophilia. Since local eosinophilia is an uncommon accompaniment to acute inflammation, perhaps the mechanism in this case involved a hypersensitivity reaction.

There is other evidence which suggests that the function of the eosinophil is closely related to the external environment they are most numerous in the skin, stomach, and small and large intestine, and their number in these tissues is very small at birth but increases markedly during the first 6 months of life [9] However if the main function of eosinophils is inactivation of histamine, it would be expected that they would be more numerous where mast cells were plentiful this situation is found in rat skin, but in the rat tongue mast cells are numerous whereas eosinophils are few and in the rat gastrointestinal tract eosinophils are numerous but mast cells are few In view of this anomaly and since the rat stomach and small intestine are rich in histamine but poor in mast cells [24] the present authors considered it possible that the many eosinophils in the rat gastrointestinal tract were there because of a high local concentration of histamine derived from the food in support of this concept, RYTÖMAA [9] stated that a meat diet increased the number of eosinophils in the alimentary tract (though no reference to the observations was given) This point was

also made by ARCHER [27] who cited the work of BIGGART [28] GODLOWSKI [29 30] and TEIR *et al.* [31] however of these authors only GODLOWSKI [30] used protein rich feeding, and although he considered that two dogs on a raw meat diet had more eosinophils in the blood and intestinal tract than did the control animals, comparison of his tables I and II indicates that the values found in the test animals were within the range found in the controls. In the present work alterations in dietary histamine and probably in endogenous histamine had no effect on the number of eosinophils present in the gastrointestinal tract.

ARCHER [2] found that an antihistamine inhibited the eosinophil response to intradermal injection of histamine in horses, thus an effect on eosinophil kinetics might have been expected when an antihistamine was continuously given to rats. The absence of such an effect, the species differences in the eosinophil response to local injection of histamine, and the finding that injection of histamine into the skin in man is followed by a local eosinophilia only in atopic individuals, suggest that the main function of eosinophils is not the inactivation of histamine.

Although ROBERTS [32] has presented evidence that eosinophils rapidly take up antigen and may thus be concerned in the formation of antibody other workers have been unable to demonstrate this effect [20 33] There is much evidence that antigen-antibody complexes are eosinotactic and are phagocytosed by eosinophils [20 25 33 34 35, 36] Thus, the main function of eosinophils is probably related to the formation or disposal of antigen-antibody complexes.

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Summary

The hypothesis that the main function of eosinophils is the inactivation of histamine was investigated by using continuous infusion of tritiated thymidine to estimate the turnover and number of eosinophils in rats given normal diet, histamine-high diet, histamine-low diet, or normal diet with antihistamine. The turnover and number of eosinophils in the bone marrow blood and tissues was not altered by any of the treatments given. These results and the known species differences in the eosinophil response to local injection of histamine suggest that the main function of eosinophils is not the inactivation of histamine.

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Cytoplasmic Shedding as a Mode of Formation of Lymphocyte-Like Blast Cells by Newt Histiocytes

E. G. Rondanelli, E. Magliulo, G. Carosi and D. Dionigi

It is well known that in haemopoietic tissues of man and animals, including amphibians, particular cells are present which, due to their small dimensions and dark staining of chromatin, are variously called 'marrow lymphocytes' 'lymphocyte-like cells' 'lymphoid blast cells' [2, 7, 8, 16, 25, 29]. Introzzi and Desylla [9], Ferrata [5], Di Guglielmo [3], Fieschi [6] and Rohr [20] had included among histiocytes a type of small lymphocyte-like cells which may be easily confused with lymphocytes for having a round compact nucleus, with a badly distinguishable nucleolus and a thin rim of slightly basophilic cytoplasm and, unlike lymphocytes, the ability to perform phagocytosis of vital dyes.

Fliedner *et al.* [7] described lymphoid blast cells appearing during haemopoietic recovery in irradiated dogs which were transfused with nitrogen-mustard treated marrow. Such cells have a large nucleus unlike that of typical lymphocytes, with a fine nuclear structure, a thin cytoplasmic rim and the capacity to synthesize DNA, thus being presumed to be endowed with stem-cell pluripotentiality. Similar cells are easily found in circulating blood of the newt *Molge Vulgaris L.* [10, 11] and are particularly numerous in early phases of the hemopoietic regeneration which ensues to refeeding the animals after a long period of starvation. Independently from any question concerning the exact role of these lymphocyte-like cells (stem cell pluripotentiality?) we have had the opportunity of observing and describing in the present paper an obsolete though not infrequent mode of formation of lymphoid cells by histiocytes which were shedding in the surrounding medium large fragments of their cytoplasm.

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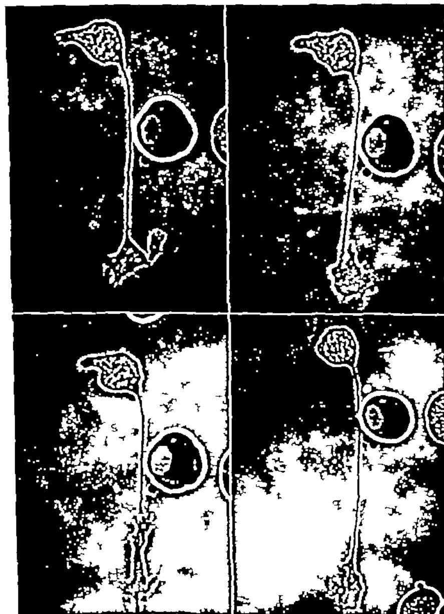


Fig 2. Progressive shedding of large cytoplasmic fragment by newt histiocyte. Frames from phase-contrast cine-recording.]

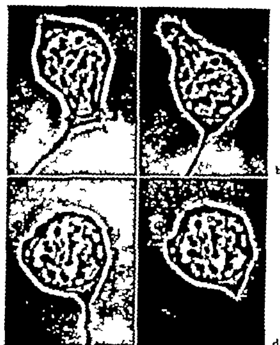


Fig. 3. Same cell as in figure 2. a–c The progressive stretching and thinning of the connecting cytoplasmic bridge causes the loss of a large portion of cytoplasm from the histiocyte. d Retraction of the remnant of the cytoplasmic bridge.

of migratory movement, but the customary shifting in the same direction of the remaining body of the cell does not follow The cell itself remains fixed in its position.

Still the pseudopodium is provided with a very marked autonomous motility appearing as it was actively pulling on its connection with the cell (fig 2) As a consequence, a progressive thinning of the reunion-trait is being operated, until the cytoplasmic bridge is broken and the pseudopodal mass is completely detached.

While the cell retracts back the remnant of the cytoplasmic bridge and acquires a round shape, with the remaining cytoplasm forming a thin rin around the nucleus (fig 3) the fragment of cytoplasm lost by the cell in the medium performs a rather intense but apparently uncoordinated locomotory activity (fig 4) The final step of the whole process has been the production of a small cell, with a high nucleo-cytoplasmic ratio still provided with histiocytic potentialities, but remarkably resembling a lymphocyte.

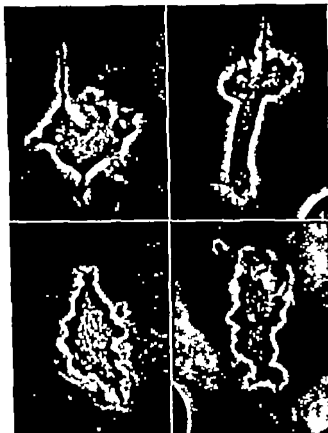


Fig 4 Fate of the portion of cytoplasm lost by the histiocyte. The detached fragment performs uncoordinated locomotory movements in the medium before dissolution.

Discussion

Cytoplasmic shedding which was first described by Ranvier [19] has already been the subject of several reviews [26 12 28, 13 17 18] the phenomenon being variously related to water and food transport or plasma formation or globulin releasing by mononuclear cells [24 26] Until now the interest of investigators was primarily focused on the probable fate of released cytoplasmic droplets and only in part on the becoming of the original cell. According to Downey and Weidenreich [4] and to Shields [26] many of the cells undergoing cytoplasmic shedding may eventually differentiate into specific cell types, namely lymphocytes, plasmocytes, etc.

Our phase-contrast cinerecords have now presented the visual evidence in living cells that such a phenomenon may actually occur at least as far as the capacity of histiocytes is concerned of assuming a lymphocyte-like appearance, and is not an artifact of fixation and staining procedures. The resulting cells, though preserving their histiocytic pattern of nuclear chromatin, may be easily confused with lymphocytes according to their dimension and the nucleo-cytoplasmic ratio. The signification of such a phenomenon is not clear but we find it very interesting that these histiocyte-derived lymphocyte-like cells were very numerous in peripheral blood of newts during early phases of post-starvation haemopoietic regeneration, and immediately preceding the appearing in the blood of large undifferentiated blast cells which probably derive from them and which subsequently differentiate into actively dividing erythroblasts.

Lymphocyte-like cells are in this rather similar to lymphoid blast cells which were regularly observed in haemopoietic organs by Magliulo [14] and Magliulo *et al.* [15] during early phases of hemopoietic regeneration in irradiated monkeys which had been transfused with autologous and homologous marrow. More recently similar reports were presented also by Thomas [27] and by Fliedner [7] strongly suggesting, though not proving, a genetic relationship between differentiated hemopoietic cells and those primitive lymphoid blast forms which we have demonstrated may in certain circumstances originate from histiocytes by means of cytoplasmic shedding. It is interesting enough that lymphocyte-like cells may also originate in peripheral blood cultures by means of the mechanism of cytoplasmic shedding from large pyroninophilic blasts [1] themselves originating from phytohemoagglutinin stimulated small lymphocytes.

Summary

In newts undergoing hemopoietic regeneration after a long period of starvation, circulating histiocytes were shown to shed large fragments of their cytoplasm, eventually differentiating into lymphocyte-like cells. The present investigation, which was conducted by means of phase-contrast cinemicrography on living cells, presents the visual evidence of an obsolete morphogenetic pattern of cells which may be provided of stem-cell pluripotentiality.

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Panmyelose mit Panhämozytopenie

H. Löffler und L. D. Leder

Unter dem Begriff «myeloproliferierende Mischformen» hat Di Guglielmo [5] solche Myelosen aus dem Komplex der myeloproliferativen Krankheiten [3] herausgehoben, bei denen mehrere Zellreihen des Knochenmarkes vom pathologischen Prozess ergriffen sind. Die Beteiligung mehrerer Zellreihen an derartigen Hämoblastosen kann entweder konstant – also während des gesamten Krankheitsverlaufes – oder nur phasenhaft nachweisbar sein. Im einzelnen unterscheidet Di Guglielmo erythroleukämische, erythromegakaryozytäre, leukomegakaryozytäre und erythroleukomegakaryozytäre Myelosen.

Beim letztgenannten Krankheitsbild, welches er auch als Panmyelose bezeichnet, beschreibt Di Guglielmo [4, 5] zwei Formen: 1. eine über Jahre verlaufende chronische, totale, hyperplastische Panmyelose, die äusserst selten sei und die sich durch eine konstante Panzythämie mässigen Grades (ähnlich der Polycythaemia vera rubra) und zusätzlich durch die Ausschwemmung von unreifen Erythroblasten, unreifen Granulozyten sowie kleinen Megakaryozytenfragmenten auszeichnet. 2. erwähnt er eine «fast stets akute, unvollständige, hyperplastische Panmyelose». Eine nähere Charakterisierung des letztgenannten Typus gibt Di Guglielmo allerdings nicht.

Die grosse Seltenheit derartiger Hämoblastosen veranlasst uns, im folgenden über einen Patienten zu berichten, dessen Krankheitsbild nach den Ergebnissen zytologischer, fermentzytochemischer und autoptischer Untersuchungen einer Panmyelose entspricht.

Kasuistik

Klinische Befunde

66jähriger Mann. 1918 Diphtherie und Ruhr. 1959 Operation eines Ösophagusdivertikels. Sonst keine ernsthaften Erkrankungen. Familienanamnese o.B.

Seit Januar 1962 bei körperlicher Belastung Atemnot und Schwindelgefühl. Ende Juli 1964 schwarzer Stuhl. Deutliche Verschlechterung des Allgemeinbefindens. Zunahme von Atemnot und Schwindelgefühl, mehrmals Ohnmachten.

Erste stationäre Aufnahme am 11.8.1964. Auffällige Blässe, reduzierter Allgemeinzustand. Leber, Milz und Lymphknoten nicht vergrößert. Keine hämorrhagische Diathese. Im Stuhl bei mehrfacher Kontrolle kein Blut nachweisbar. Laborbefunde: BSG 25/60 mm. Serumtransaminasen, alkalische Phosphatase, Bilirubin, Serumlabilitätsproben, Gesamtprotein und Elektropherogramm unauffällig. Röntgenologisch zwei Divertikel im unteren Sigma. Magen-Darm-Trakt sonst o.B. Weiterhin schwere normochrome Anämie, Leukozytengesamtzahl an der unteren Normgrenze. Therapie: Insgesamt 9 Bluttransfusionen zu je 500 ml. Anstieg des Hämoglobins von 5,3 auf 11,4 g%, BSG 11/28 mm. Am 11.9.1964 Entlassung.

Erneut Hospitalisierung bei im wesentlichen unverändertem klinischem Befund wegen Zunahme der subjektiven Beschwerden am 21.10.1964. Röntgenologisch an Ösophagus, Magen, Colon, Nieren, Gallenblase und Thoraxorganen kein pathologischer Befund. Hämoglobin 7,9 g%. Leukozytengesamtzahl zwischen 3100 und 6000/mm³ schwankend. Thrombozyten 59000/mm³. Serum-Eisen 216–319 μg%. HbE 30,6. Retikulozyten 2–10 ‰. Osmotische Resistenz der Erythrozyten 0,30%–0,34% NaCl. Gerinnungsuntersuchungen

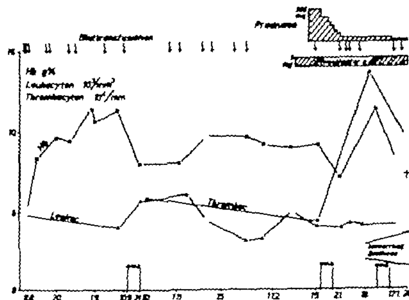


Abb. 1 Verhalten von Hämoglobin, Leukozyten und Thrombozyten während des Krankheitsverlaufes.

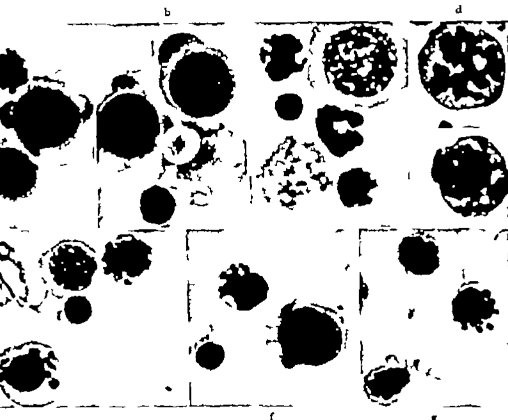


Abb. 2. (a) und (b) Junge, megaloblastäre Erythroblasten mit pseudopodienartigen Plasmaausläufern. (c) Pathologische Erythroblastenmitosen mit erklumpten Chromosomen. Links unten ein Promyelozyt. (d) bis (g) Pathologische Erythroblastenmitosen sowie Erythroblasten mit paranukleären Chromatinbrocken. Sternalpunktat. Pappenheim-Färbung (×1400).

Quick 108%, Gerinnungszeit 2'30" Blutungszeit 15 Faktor II 70%, Faktor V 114%, Faktor VII 86%. Über das Verhalten von Hämoglobin, Leukozyten und Thrombozyten während des Krankheitsverlaufes unterrichtet die Abb. 1. Die Sternalpunktion (28.10.1964 und 7.12.1964, siehe unten) ergab die Diagnose Panmyelose. Therapie: Ab 8.12. täglich 2,5 mg Amethopterin sowie Prednison mit einer anfänglichen Dosis von 200 mg/die, die kontinuierlich bis auf 25 mg/die reduziert wurde. In der Zeit vom 30.10.–14.12.1964 insgesamt 7 Bluttransfusionen zu je 500 ml. Bei der Entlassung am 15.12. Hämoglobin 9,1 g%, Leukozyten 4000/mm³, Thrombozyten 42000/mm³.

Dritte stationäre Aufnahme am 2.1.1965. Hb 7,0 g% bei 2,33 Mill. Erythrozyten, Leukozyten 2900/mm³. Serumeisen 182 und 217 μg%. Weiterbehandlung mit täglich 25 mg Prednison sowie 2,5 mg Amethopterin. Außerdem in der Zeit vom 2.1.–14.1.1965 insgesamt 4 Bluttransfusionen zu 500 ml. Entlassung am 14.1.1965.

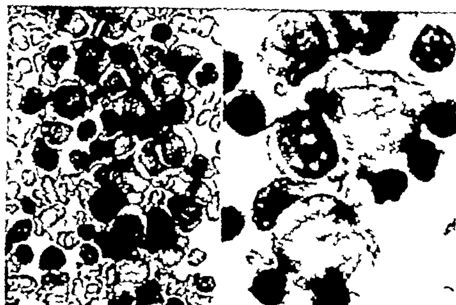


Abb.3 (a) Blasten, Promyelozyten und einzelne sogenannte Pseudopelgerformen. (b) Blasten, Promyelozyten, dazwischen Erythroblasten mit stark verklumpten und verdichteten pyknoseartig veränderten *Kernen*. Sternalausstrich (× 1400)

Vierter stationärer Aufenthalt in der Zeit vom 17 1 1 1 1965 wegen Fieberschüben und ausgedehnten flächenhaften Hautblutungen. Schwere, durch antibiotische Behandlung und Herzkreislauftherapie nicht mehr zu beherrschende Bronchopneumonie. Am 21 1 1963 Exitus letalis.

Morphologische Befunde

Im *peripheren Blutausstrich zeigten die Erythrozyten eine hochgradige Anisozytose* und Poikilozytose sowie eine ausgeprägte Hypochromasie. Häufig waren sie basophil punktiert und enthielten Cabotsche Ringe. Auf 100 *Leukozyten* fanden sich maximal 2 Normoblasten, deren Zytoplasma vakuolisiert, zum *Teil* optisch leer erschien. Im weissen *Differentialblutbild* wurden maximal 3% Myeloblasten gezählt. Der *Anteil* der Lymphozyten lag im Normbereich. Die neutrophilen Granulozyten waren vermindert und zum grossen Teil pathologisch verändert. Es fanden sich sogenannte *Pseudopelgerformen*, weiterhin vereinzelt übersegmentierte und polyploide Neutrophile mit eigenartig verklumpten Kernen. Gelegentlich waren im Zytoplasma der stab- und segmentkernigen Neutrophilen Vakuolen und Doehle-Körperchen vorhanden. Auch die Thrombozyten waren atypisch. Sie zeigten eine erhebliche Polymorphie, und es fanden sich Riesenplättchen sowie ungranulierte Formen.

Bei den beiden *Sternalpunktatuntersuchungen*, die wir am 28.10. und am 7.12.1964 durchführten, wurden so weitgehend identische Veränderungen gefunden, dass wir sie zusammenfassend darstellen. Die Ausstriche waren recht zellreich. An der Zellvermehrung waren alle drei blutbildenden Zellreihen beteiligt. Am stärksten trat die Erythropoiese hervor. *Es fanden sich zahlreiche Anomalien, von denen alle Reifungsstufen betroffen waren* (Abb.2): *megaloblastäre* Formen, zwei- und mehrkernige Erythroblasten, atypische

Mitosen sowie sehr häufig Kernverklumpungen und Chromatinabsprengungen. Bei manchen Erythroblasten war das Zytoplasma von kleinen runden Chromatinbrocken angefüllt. Solche Veränderungen waren manchmal kaum von pathologischen Mitosen zu unterscheiden. Manche reiferen Erythroblasten zeigten ein auffällig schmales Zytoplasma, andere eine Vakuolisierung. Auch bei den reiferen Erythroblasten waren die Kerne verändert, und zwar bekamen sie häufig ein dichtes, klumpiges, fast strukturloses Chromatin, so dass der Eindruck einer Pyknose entstand (Abb. 5b)

In der granulozytären Reihe fielen insbesondere Blasten und Promyelozyten auf (Abb. 5b) die herdförmig zusammenlagen und erhebliche Kernpolymorphien und Granulationsanomalien aufwiesen. Die reiferen Granulozytenvorstufen entsprachen zum Teil sogenannten Pseudopelgerzellen (Abb. 3a) vereinzelt sah man Riesenformen, wie sie bei megaloblastärer Anämie auftreten.

Die reichlich vorkommenden Megakaryozyten (Abb. 4) liessen erhebliche Grössendifferenzen erkennen. Zytologisch fanden sich zahlreiche Atypien und Abweichungen von der Norm. Neben Megakaryozyten mit stark segmentierten oder gelappten Kernen sahen wir Knochenmarkriesenzellen mit sehr stark verklumptem Chromatin, ähnlich wie bei einer Kernpyknose. Das Zytoplasma der Megakaryozyten war in den meisten Fällen basophil und nicht granuliert sowie von verschieden grossen Vakuolen durchsetzt. Die Zellgrenzen waren oft nicht klar erkennbar sondern stark zerklüftet, so dass der Eindruck eines scholligen Plasmazerfalls entstand. Innerhalb von basophilen Zytoplasmabezirken sah man schliesslich ungefärbte oder zart rötlich gefärbte Areale. Auch bei den Megakaryozyten fielen uns zahlreiche Mitosen mit Chromosomenabsprengungen und Chromatinverklumpungen auf.

Cytochemische Befunde

Eisenreaktion. 60% der Erythroblasten der Sternalausstriche enthielten freies Eisen in Form von kleinen Granula. Dabei waren die Granula gegenüber der Norm vergröbert sie lagen meist perinukleär Weiterhin war eine erhebliche Vermehrung von eisenspeichernden Retikulumzellen zu konstatieren.

Perjodsäure-Schiff-Reaktion (PAS) I 12% der Erythroblasten des Knochenmarkes fand sich eine schwache diffuse positive Reaktion. Die Megakaryozyten waren dagegen häufig negativ zum Teil auch schwach homogen positiv Von den Zellen der Granulozytopoese reagierten die Blasten negativ in den Promyelozyten und den weiteren Ausreifungsstadien fiel die Reaktion zwar positiv aus, aber schwächer als normal.

Alkalische Phosphatase. In den Sternalausstrichen zeigten Kapillarendothelien sowie einzelne reife Granulozyten eine positive Reaktion. Im peripheren Blutbild (Untersuchung vom 7 12. 1964) waren 38% der reifen Granulozyten phosphatase-positiv wobei ein Index von 44 ermittelt wurde

α-Naphthylacetat-Esterase. Am stärksten positiv reagierten die Retikulumzellen. Bei den Erythroblasten aller Reifungsstadien war die Aktivität ganz erheblich gesteigert, wobei das Reaktionsprodukt diffus im Zytoplasma verteilt erschien. Auch die Erythroblastenmitosen wiesen diese Aktivierungssteigerung auf. In den Megakaryozyten war gelegentlich eine ausserordentlich grobgranuläre Ablagerung von Reduktionsprodukt vorhanden. Die Granulozytopoese zeigte keine Besonderheiten.

Autoptische Befunde (Sekt.-N 73/65)

Makroskopische Befunde Panmyelose: Dunkelrotes Mark im gesamten Femur In den Wirbelkörpern und im Sternum. Geringgradige Vergrösserung von Leber und Milz. Hämorrhagische Diathese: Bis reiskornpfennigstückgrosse Blutungen in der Magenschleimhaut: flächenhafte Blutungen in der Nierenbeckenschleimhaut beiderseits und im Nierenparenchym rechts pfennigstückgrosse, subintimale Blutung im Bereich der Aorta abdominalis; flächenhafte Hautblutungen im Bereich des Stammes und der Extremitäten; kleine nicht ganz frische

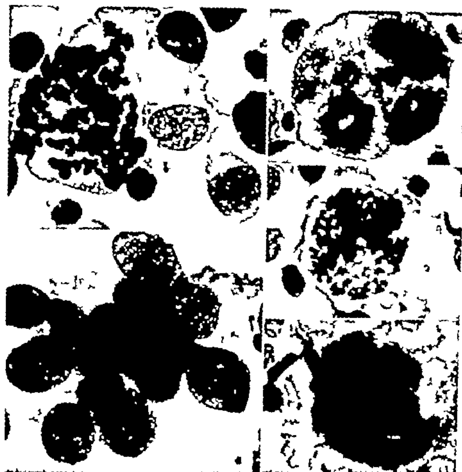


Abb. 4. Atypische Megakaryocyten und Megakaryocytenmitosen. Sternalausstrich. Pappenheim-Färbung (× 1000)

Hirnblutungen im Putamen-Klaustrumbereich links. Schwere chronische katarrhalische Tracheobronchitis. Geringgradige diffuse Bronchiektasen beiderseits. Schwere fibrinreiche Pneumonie beiderseits. Fibrinöse Pleuritis beiderseits, rechts stärker als links. Mäßig schweres chronisches substantielles Lungenemphysem. Dilatation der rechten Herzkammer. Leberverfettung. Lipoidentspeicherung der Nebennierenrinde beiderseits. Geringgradige Arteriosklerose der Aorta. Schwere Coronararteriensklerose mit weitgehendem Verschluss des R. interventricularis der A. coronaria sinistra. Dilatation der linken Herzkammer. Schweres Lungenödem. Cholesteatose der Gallenblase. Adenomatöse Prostatahyperplasie. Geringgradige Balkenblase.

Histologische Befunde. Im *Knochenmark* von Wirbel, Sternum und Femur verhielt sich die Spongiosa regelhaft, die Silberfasern waren nicht vermehrt. Die Fettzellen waren fast vollständig geschwunden. Bei schwacher Vergrößerung war bereits eine merkwürdige und ungewöhnliche Vielfalt von Zellen auffällig: Neben sehr kleinen kamen sehr große, neben

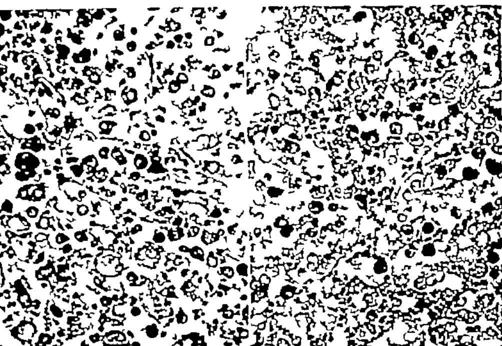


Abb. 5. Schnittpräparat vom Femurmark. ( ) Schwund der Fettzellen. Starke Vermehrung von atypischen Megakaryocyten und zum Teil mehrkernigen Erythroblasten. Giemsa. (b) Nachweis der Naphthol-AS-D-Chloracetat-Esterase am Paraffinschnitt. Die atypischen, promyelocytenähnlichen Zellen der neutrophilen Granulopoiese heben sich durch ihre positive Reaktion (schwarz, im Original leuchtend rot) heraus (×350)

stark basophilen oxyphile Elemente mit teils runden, teils auch bizarren, gelappten Kernen vor. Viele der Zellen waren mehrkernig.

Bei stärkerer Vergrößerung (Abb. 5a) waren Megakaryocyten, Erythroblasten und Granulopoesezellen zu unterscheiden. Die Megakaryocyten lagen in lockeren Gruppen und besaßen häufig sehr weites, teils basophiles, teils oxyphiles, leicht gekörntes Plasma. Oft waren ihre Plasmaränder ausgesprochen unscharf begrenzt. Die Megakaryocytenkerne zeigten ein lockeres, helles Chromatingerüst und waren oft bizarr geformt mit Lappungen und Segmentierungen aller nur erdenklichen Formen. Nukleolen waren deutlich erkennbar, die Kernmembranen meist scharf ausgeprägt.

Die Erythropoesezellen standen zahlenmäßig durchaus im Vordergrund. Es waren alle Reifungsstufen bis zum oxyphilen Normoblasten in bunter Mannigfaltigkeit zu erkennen, doch bestanden erhebliche Größenunterschiede. Während die Normoblasten durch ihr oxyphiles Plasma relativ gut erkennbar waren, gelang die sichere Identifizierung von unreifen basophilen Erythropoesevorstufen selbst im Giemsa-Präparat nicht in allen Fällen. Unter den unreifen Erythropoesezellen fielen häufig zweikernige Exemplare auf. Viele Erythropoesezellen waren in Mitose begriffen. Dabei fanden sich im Plasma nicht selten abgesprengte Kernteile. Besonders auffällig war das Fehlen der normalerweise immer vorhandenen und für die Erythropoese typischen betont gruppenförmigen Lagerung der Zellen.

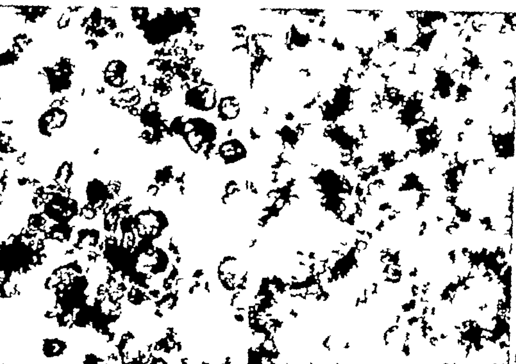


Abb. 6. Schnittpräparat vom Femurmark. (a) Nachweis der α-Naphthylacetat-Esterase: Atypische Erythroblasten stark positiv mit typischer perinukleärer Reaktion (schwarz, im Original rotbraun). Dazwischen einige sehr stark positive Plasmaausläufer von Retikulumzellen (×560). (b) Nachweis der sauren Phosphatase. Im wesentlichen sind die Retikulumzellen positiv (*schwarz, im Original rot*), daneben wenige *Proerythroblasten mit punktförmiger* paranukleärer Reaktion (z.B. rechts oben) (×350).

Die Granulopoiesezellen traten im Präparat zahlenmäßig zurück. Segmentkernige, stabkernige Metamyelocyten und Myelocyten waren kaum vorhanden. Dagegen konnten einzeln liegende atypisch gestaltete Promyelocyten identifiziert werden. Auch Vorstufen der Eosinophilen kamen gelegentlich vor.

Weiterhin waren Retikulumzellen *in mäßiger* Anzahl, einige Plasmazellen sowie *Lymphocyten eingestreut. Gewebsmastzellen kamen nicht vor.*

In der *Milz* fanden sich innerhalb der Sinus nur wenige *Infiltrate* aller drei blutbildenden Zellsysteme. Am auffälligsten stachen die Megakaryocyten hervor, die die gleichen pathologischen Veränderungen zeigten wie im Knochenmark. Im Gegensatz zu den Verhältnissen im Knochenmark waren unter den Myelopoiesezellen relativ reichlich reife Granulocyten vorhanden. Erythropoiesezellen kamen nur vereinzelt vor. Die Pulparetikulumzellen wiesen eine starke Hämosiderose auf. Plasmazellen waren reichlich vorhanden.

In der *Lunge* bestand eine erhebliche fibrinreiche Bronchopneumonie mit Infiltraten durch gelapptkernige neutrophile Granulocyten, von denen ein größerer Teil als Pseudo-*pelgerformen vorlag.* In den Alveolen waren außerdem große Mengen einer eiweißreichen Flüssigkeit im Sinne eines *Lungenödems vorhanden.* Vielfach traf man auf kleine Blutungsherde.

I Leber, Lymphknoten vom Lungenhilus, *Tonsillen*, Niere und *Herz* ließen keine Besonderheiten, insbesondere keine leukämischen Infiltrate

Histochemische Befunde Im *Knochenmark* reagierten die Megakaryozyten bei der PAS-Färbung sämtlich schwach diffus-positiv. Die Reaktion war jedoch erheblich schwächer als normalerweise. Die Megakaryozyten zeigten weiterhin eine schwache Reaktion beim Nachweis von α-Naphthylacetat-Esterase, von Naphthol-AS-acetat-Esterase sowie von saurer Phosphatase. Naphthol-AS-D-Chloracetat-Esterase und alkalische Phosphatase besaßen die Megakaryozyten nicht.

Die Erythropoiesezellen reagierten beim α-Naphthylacetat-Esterase-Nachweis außerordentlich stark positiv (Abb. 6a) und erreichten fast den Aktivitätsgrad der Retikulumzellen, die die am stärksten positiven Knochenmarkzellen darstellen. Besonders intensiv färbten sich große, den Proerythroblasten an die Seite zu stellende Elemente an. Sie zeigten bereits nach einer Inkubationszeit von nur 2 min eine deutliche Reaktion. Im Unterschied zum Sternalpunktat war die Fermentaktivität vor allem auf eine schmale, den Kern unmittelbar wie eine Schale umgebende Plasmazone konzentriert, wie dies für normale Erythropoiesezellen hochcharakteristisch ist. Beim Nachweis der sauren Phosphatase reagierte ein kleiner Teil der besonders unreifen Erythroblasten mit einer deutlichen Anfärbung innerhalb eines paranukleären Plasmabezirkes (Abb. 6b). Naphthol-AS-acetat-Esterase, Naphthol-AS-D-Chloracetat-Esterase und alkalische Phosphatase konnte in den Erythropoiesezellen nicht nachgewiesen werden.

Mit dem Nachweis der Naphthol-AS-D-Chloracetat-Esterase stellten sich die neutrophilen Granulopoiesezellen elektiv dar. Sie kamen (Abb. 5b) nur in relativ geringer Anzahl vor und entsprachen etwa den Promyelozyten. Abweichend von den normalen Promyelozyten besaßen sie jedoch unregelmäßig gestaltete Kerne. Manchmal waren sie auch zweikernig. Beim Nachweis der α-Naphthylacetat-Esterase, der Naphthol-AS-acetat-Esterase, der sauren und der alkalischen Phosphatase verhielt sich die Granulopoiese unauffällig.

An den übrigen Organen konnten durch die zytochemischen Untersuchungen keine Befunde erhoben werden, die über diejenigen Gesichtspunkte hinausführten, die bereits durch die histologische Untersuchung gewonnen worden waren.

Diskussion

Die klinischen Befunde unseres Patienten erlaubten zunächst ohne die Untersuchung des Sternalpunktates keine endgültige Diagnose. Zwar bestand von Beginn der Erkrankung an eine Panhämozytopenie mit Anämie, Thrombozytopenie und Leukozytenwerten, die an der unteren Normgrenze lagen oder gering erniedrigt waren, und im Differentialblutbild wurden einzelne Blasten und Erythropoiesezellen gefunden. Doch wiesen diese Befunde, wie auch die qualitativen Veränderungen der neutrophilen Granulozyten mit Pseudopelgerformen, Übersegmentation und Polyploidie und wie die morphologischen Veränderungen der Erythrozyten und Thrombozyten lediglich auf eine schwere Schädigung der Hämatopoese hin, ohne eine sichere Diagnose zu erlauben.

Erst bei der Untersuchung des Sternalmarkes konnte die Erkrankung in ihrem Wesen klar erkannt werden. Es fand sich eine schwere Hyperplasie aller drei blutbildenden Zellreihen mit hochgradigen

Atypien in der Erythropoese, mit atypischen Blasten und Promyelozyten in der Granulopoiese und vor allem mit reichlich polymorphen, unreifen und atypischen Megakaryozyten. Die Erythroblasten und die Megakaryozyten fielen besonders auf. Einzelne Erythroblastenkerne waren fragmentiert, neben noch erhaltenen Kernen fanden sich Ansammlungen von «Chromatinkugeln» und ausserdem sah man sowohl in den Erythroblasten als auch in den Megakaryozyten zahlreiche verschiedengestaltige Mitoseanomalien. Zytochemisch imponierte eine deutliche Vermehrung der Sideroblasten mit vergröberten Eisengranula. Ferner war die α-Naphthylazetat-Esterase-Aktivität der Erythroblasten erheblich gesteigert. Bei der PAS-Reaktion ergab sich allerdings keine wesentliche Erhöhung des Anteiles positiver Erythroblasten gegenüber der Norm. An Hand dieser Befunde konnte die Diagnose einer Panmyelose sicher gestellt werden.

Bei der Autopsie wurde eine eindeutige Hyperplasie des Knochenmarksparenchyms mit einem fast vollständigen Schwund der Fettzellen festgestellt. Ausserdem fanden sich in der Milz Infiltrate von Megakaryozyten, von Granulopoiesezellen und von Erythroblasten. Im Knochenmarkschnitt fiel vor allem die ungewöhnliche Vielfalt der Zellen und die sehr starke Vermehrung von atypischen Megakaryozyten auf, die an manchen Stellen sehr dicht gelagert waren. Zahlenmässig beherrschte jedoch – wie im Knochenmarkausstrich auch – die hochgradig polymorphe und atypische Erythropoiese mit vielen mehrkernigen Zellen und reichlich Mitosen das Bild. Die Granulopoiese stand dagegen quantitativ zurück, war aber auch im histologischen Schnitt erkennbar pathologisch verändert und insbesondere durch die Anwendung der Naphthol-AS-D-Chlorazetat-Esterase-Reaktion am Paraffinschnitt [10] gut darzustellen. Damit war auch in Anbetracht der autoptischen Befunde an dem Vorliegen einer Hämoblastose nicht zu zweifeln, wie aus der schweren Infiltration des Knochenmarkes mit Verdrängung der Fettzellen und aus dem Nachweis von atypischen Megakaryozyten, Granulopoiesezellen und Erythropoiesezellen in der Milz hervorgeht. Dass es sich im vorliegenden Falle zweifellos um eine Panmyelose handelt, zeigen die schweren morphologischen Veränderungen an allen drei Knochenmarkszellreihen.

Ausser morphologischen Befunden, die das Vorliegen eines malignen Geschehens bewiesen, waren auch entsprechende zytochemische Befunde vorhanden, wie sie bei anderen malignen Hämoblastosen vorkommen. So imponierte eine deutliche Vermehrung von Sidero-

blasten mit vergröberten Eisengranula sowie eine Vermehrung von eisenspeichernden Retikulumzellen. Beide Befunde sind als Ausdruck einer Eisenverwertungsstörung zu betrachten und sie kommen – abgesehen von der sideroachrestischen Anämie – auch bei Erythrämien und Erythroleukämien vor [7 15 8] Im übrigen ist die bei unserer Beobachtung vorliegende Eisenverwertungsstörung im Rahmen einer Abhandlung über die pathologische Anatomie der sideroachrestischen Anämie ausführlicher besprochen [12] so dass sich ein weiteres Eingehen auf diese Problematik erübrigt.

Die erheblich gesteigerte α Naphthylazetat Esterase Aktivität der Erythroblasten stützt ebenfalls die Annahme einer malignen Entartung der roten Reihe, da ähnliche Aktivitätserhöhungen bisher nur bei Erythrämie und Erythroleukämie beobachtet wurden wenn man von der perniziösen Anämie absieht [16 13 14 11] Offenbar ist dieser zytochemische Befund immer dann zu erheben, wenn eine in hohem Masse ineffektive Erythropoiese vorliegt.

Die PAS-Reaktion erbrachte allerdings keinen Hinweis auf das Vorliegen einer malignen Veränderung innerhalb der Erythropoiese denn der Anteil positiver Erythroblasten war gegenüber der Norm nicht wesentlich erhöht. Im allgemeinen wird eine deutlich gesteigerte PAS-positive Reaktion eines grossen Teiles der Erythroblasten als sehr charakteristischer Befund bei reiner Erythrämie und bei Erythroleukämie angesehen [1 18, 15] Dies konnten wir bei eigenen Untersuchungen an 6 Patienten mit Erythroleukämie und an einem Patienten mit erythrämischem Terminalstadium bei chronischer myeloischer Leukämie bestätigen Bei subakut oder chronisch verlaufenden Neoplasien der Erythropoiese kann die gesteigerte PAS-Reaktion der Erythroblasten aber auch fehlen oder nur sehr gering ausgeprägt sein [22, 7] Daraus ergibt sich, dass nur der positive Befund als Stütze für die Annahme einer malignen Erkrankung der roten Reihe anzusehen ist, während umgekehrt ein negativer Befund wie er in unserem Falle erhoben wurde, eine solche Möglichkeit keineswegs ausschliesst.

Bei dem von uns beschriebenen Krankheitsbilde handelt es sich um eine besondere Hämoblastose die von anderen myeloproliferativen Erkrankungen, wie der Polycythaemia vera rubra der Osteomyelosklerose und Myelofibrose einschliesslich der Übergangsformen zu chronischen myeloischen Leukämien und von der sogenannten Panmyelopathie mit hyperplastischem Mark abgegrenzt werden kann Von diesen Krankheitszuständen unterscheidet sich unsere Panmyelose durch die sehr schweren Zellatypien. Von der Osteomyelo-

sklerose und von der Myelofibrose ist die Panmyelose durch die fehlende Spongiosa- und Faservermehrung abgegrenzt. Gegen das Vorliegen einer Polycythaemia vera rubra sprechen die klinischen Befunde. Eine Panmyelopathie mit hyperplastischem Mark, wie sie in extrem seltenen Fällen als Initialstadium einer chronischen Benzolvergiftung beschrieben ist [2], kann anamnestisch ausgeschlossen werden. Andere Fälle von Panmyelopathie, die meist ätiologisch nicht zu klären sind und vielfach als Autoimmunreaktion gedeutet werden, kommen ebenfalls nicht in Frage, da bei einer solchen Annahme die schweren zytologischen und zytochemischen Veränderungen unverständlich blieben, die eindeutig auf eine maligne Proliferation der Knochenmarkszellen hinweisen. Im übrigen kann keinesfalls ausgeschlossen werden, dass sich hinter manchen Fällen von derartigen Panmyelopathien mit hyperplastischem Mark in Wirklichkeit Panmyelosen verbergen.

Die Befunde des von uns beobachteten Patienten weichen in verschiedenen Punkten von der Beschreibung des Panmyelosetypus I von Di Guglielmo [5] ab. So fehlt z.B. die Panzythämie, und unreife Zellen der Hämopoiese wurden nur bei längerem Suchen in geringer Zahl im peripheren Blutbild gefunden. Schliesslich erstreckte sich der Krankheitsverlauf auch nicht wie bei den Fällen Di Guglielmos über mehrere Jahre, sondern war sehr kurz. Ob eine Ähnlichkeit mit der zweiten von Di Guglielmo erwähnten Verlaufsform der Panmyelose besteht, die er eine «fast stets akute, unvollständige, hyperplastische Panmyelose» nennt, vermögen wir nicht zu entscheiden, da Di Guglielmo eine genaue Charakterisierung dieses Krankheitsbildes nicht gegeben hat.

Von manchen werden unter dem Terminus Panmyelose Krankheiten verstanden, die von unserer Beobachtung in vielen Einzelheiten abweichen und die im allgemeinen auch nicht ohne weiteres als maligne Hämoblastosen angesehen werden. So belegen Heilmeyer (?) Herreuval und Mitarbeiter [9] die Osteomyelosklerose und verwandte Krankheitsbilder mit dem Ausdruck «panmyélémie» und Rappaport [19] fasst unter dem Begriff Panmyelose die Polycythaemia vera und die «Myelosklerose mit myeloischer Metaplasie» zusammen. Wir sind dagegen der Meinung, dass die Bezeichnung Panmyelose solchen Hämoblastosen vorbehalten bleiben sollte, die eindeutig Zeichen einer malignen Proliferation aller drei blutbildenden Systeme in Form von schweren Zellatypien etc. aufweisen, wie das bei unserer Beobachtung der Fall ist. Im übrigen ist das von uns beschriebene Krankheitsbild

durch den völlig abweichenden klinischen Verlauf und den abweichenden den pathologisch-anatomischen Befund sowohl von der Osteomyelosklerose als auch von der Polycythaemia vera klar abgetrennt.

Unsere Beobachtung könnte die durch zytogenetische Untersuchungsergebnisse gestützte Ansicht von einer gemeinsamen Stammzelle für die Erythropoiese, die Granulopoiese und die Megakaryozytopoiese weiter unterbauen [20, 21, 23], eine Ansicht, die u. a. aus dem Vorkommen des für die chronische myeloische Leukämie typischen Philadelphia-Chromosoms auch in Erythroblasten und Megakaryozyten abgeleitet ist. Eine solche gemeinsame Stammzelle wird auch von HAYHOE und Mitarbeitern [6] anhand zytologischer und zytochemischer Untersuchungsergebnisse bei unreifzelligen Leukosen diskutiert. Allerdings ist auch die Möglichkeit zu erwägen, dass Knochenmarkszellen – vor allem, da sie genetisch untereinander nahe verwandt sind – gleichartig anfällig gegenüber geschwulstauslösenden Noxen sind. Damit wäre z. B. denkbar, dass ein solches Agens bei allen Zellreihen gleichartige Veränderungen hervorruft, ohne dass eine gemeinsame Stammzelle unbedingt vorliegen muss.

Zur Frage der Therapie ist zu erwähnen, dass wir mit kleinen Dosen von Amethopterin, wie sie von MOESCHLIN [17] zur Behandlung von Erythroleukämien empfohlen werden und wie sie auch von uns mit gutem Erfolg bei einem Fall von Erythroleukämie eingesetzt werden konnten, in Kombination mit Prednison bei unseren Patienten keinen sichtbaren Erfolg hatten. Allerdings konnten wir die Behandlung nur 14 Tage lang durchführen. LÜDIN [13a] hat bereits 1950 einen deutlichen Effekt des Folsäureantagonisten Aminopterin bei Erythroleukose beschrieben.

Zusammenfassung

Es wird über einen 66jährigen Patienten berichtet, der das Krankheitsbild einer Panmyelose mit Panhämocytopenie bot. Bei der bioptischen und autoptischen Untersuchung wurde eine schwere Hyperplasie des Knochenmarkes festgestellt, an der die Erythropoiese, die Granulopoiese und die Megakaryozytopoiese beteiligt waren. Alle drei Zellreihen wiesen überdies schwere zytologische Atypien auf. In der Milz fanden sich extramedulläre Blutbildungsherde. Die zytologischen und zytochemischen Befunde werden im Hinblick auf die allgemeine hämatologische Bedeutung solcher Fälle besprochen, und es werden diagnostische, differential-diagnostische und therapeutische Fragen gestreift.

Summary

This paper reports a case of malignant panmyelosis with panhemocytopenia, which occurred in a 66-years-old patient. At biopsy and autopsy the bone marrow revealed a heavy hyper

plasia of the blood forming parenchyma including erythropoiesis, granulopoiesis, and megacaryopoesis as well. There were in addition, many cytological atypias in each of the three bone marrow cell strains. The cytological and cytochemical observations are discussed with respect to the general hematological importance of such cases, and problems of diagnosis, differential diagnosis, and therapy are briefly mentioned.

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Fatal Peroral Iron Poisoning in a Young Woman

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During recent years a considerable number of severe or fatal accidental poisonings by iron salts has been reported [1 2, 3 5 6 7 8, 12 13 14] In the USA approximately 2000 cases of iron intoxication are encountered yearly with a death rate of 45 % [4] In most cases children ingested tablets of ferrous salts, usually coloured or coated with chocolate. The exact dose of iron ingested could only be determined accurately in 36% of a group of 172 children [4] The introduction of deferoxamine changed the treatment and improved the fatal outcome of iron poisoning [10] The death rate is now lower than 10 % if proper therapy is started early enough The normal low iron excretion in urine is increased by deferoxamine to levels exceeding one hundred times the normal values [10 15]

The majority fo cases with iron poisoning reported were in children and it may be of interest to report a case of suicidal iron intoxication in an adult with a fatal outcome

Case Report

A 23-year-old woman was admitted to the Psychiatric Department with the diagnosis of psychoneurosis. Her fiancé reported that he had found her in a semicomatous state vomiting brown and black masses which were suggestive of gastric hemorrhage. Thereafter the patient admitted to have ingested approximately 100 tablets 2 days previously because of conflicts in her family On admission the patient was semicomatous and said with weak voice that she took tw bottles of antianemic tablets about 52 h before The local physician reported later that he found two empty bottles of antianemic tablets, containing 50 mg iron each, in the patient room. The total dose taken was 10 g iron or 166 mg/kg body weight The clinical findings were dark brown ppearance of skin similar to sun tanning Ptosis of the lids was found, but the reflexes on examinations of the extremities were normal. Skin temperature was 36.5°C, the blood pressure was not measurable, the pulse rate 130.

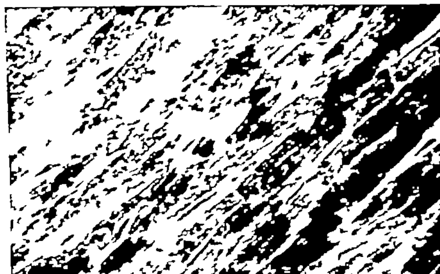


Fig. 1 Myocardium. Sudan III Myocardial fibers with microscopic droplets of fat (appearing as black spots).

Hb 72.2% (11.53 g %) RBC 3,830,000, CI 0.93, WBC 49,000, bands 1 %, segm. 81%, lymph. 12% monocyt. 6%. Blood urea 84 mg %, Iron 480 μg %. Stool examination for occult blood positive.

After infusion of Ringer solution with Dexamethasone Rheomacrodex and antibiotic therapy the blood pressure rose to 70/60 mm Hg. The patient was then transferred to the Intensive Care Unit, where she died after admission.

Investigations (Intensive Care Unit) Blood urea 84 mg %, blood sugar 110 mg %. pH 7 133; 7 163; HCO_3 12.4 10.4 mEq/l total CO_2 13.5 11.3 mEq/l, physic. bound CO_2 1 12 0.9 mEq/l pCO_2 38.30 mm Hg, HCO_3/CO_2 1 10.9 1 11.6. Electrophoresis Alb. 62.5%, α_1-glob. 4.8%, α_2-glob. 6.4 %, β-glob. 8.8%, γ-glob. 18.5%.

Post mortem examination. The body is that of an adequately nourished woman. Both lungs show considerable degree of congestion and numerous hemorrhages especially on the anterior border I the pleural cavities approximately 100 ml of hemorrhagic fluid were found. The heart, pericardium and thymus ppeared normal and so did the gallbladder and pancreas. Lateral to both adrenals and under the capsules of the kidneys extensive hemorrhages were found. The kidneys ppear pale with normal ureters; the bladder which is also normal, contains no urine. The reproductive organs are normal as are the brain and meninges. The stomach shows an area of congestion containing numerous small hemorrhagic spots with some edema. The stomach contains some millilitres of dark brown coffee-ground material. In the duodenal area there are abundant hemorrhages. The small intestine contains dark tarry material especially in the ileum. The large intestine shows areas of congestion and swelling with hemorrhagic spots. The liver is pale, flabby greasy to touch, with areas of orange and yellow color The spleen is normal in size, weight and consistence.

Histology The heart shows fatty degeneration (fig. 1) as do also the epithelial cells of the tubules in the kidneys (fig 2). In the liver widespread and uniform fatty change was found. The droplets of fat in the cells are confluent, so that no protoplasm can be seen (fig 3)

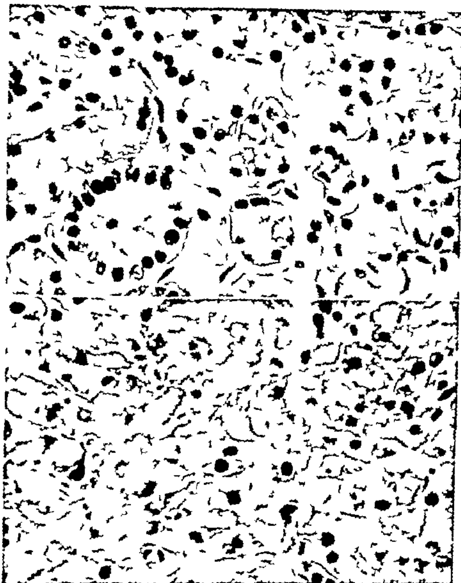


Fig. 2. Kidney. Haematoxyline-eosine. Numerous vacuolized tubular cells after extraction of fat.

Fig. 3. Liver. Haematoxyline-eosine. Heavy parenchymatous degeneration of liver cells and deposition of fat.

Staining with Sudan III shows the fatty degeneration to be of a higher degree than expected by the hematoxyline-eosine method. The main lesions occur in the gastro-intestinal tract. In the stomach minimal changes were found, but in the duodenal mucosa severe necrosis of

the epithelium existed, so that no normal areas were encountered. In the deep layers the cell nuclei were colored pale or bent. The muscularis mucosae was edematous and thickened, the Brunner cells were weakly colored. It is of interest to note that in the tubules of kidneys and hepatic cells iron could not be demonstrated.

Discussion

REISSMANN *et al.* [9] studied the mechanism of the toxic action of iron in animals. By stomach or duodenal tube or by enemas dissociable iron salts were given. The lethal dose in dogs and rabbits was found to be approximately 150 to 200 mg iron per kilogram body weight. In the majority of the animals no histologic changes were seen in the intestinal mucosa, but in some instances intestinal bleeding from congested capillaries occurred. In children who died from iron intoxication, necrosis of the intestinal mucosa was encountered, although in some cases minimal necrotic changes were found. REISSMANN estimates that the heavy intestinal necrosis in children is due to the corrosive effect of iron salts in substance while in the experiments with dogs and rabbits aqueous solutions have been used.

Poisoning with iron in adults is very rare. The patient reported passed at home of the three characteristic phases of iron intoxication 1 Acute gastro-intestinal symptoms, 2. relative wellbeing 3 fatal termination. She entered the hospital in the terminal phase of intoxication and died shortly after admission so that no therapy could be given. It appears that once the gastric mucosa is damaged, iron escapes from the gut into the tissues and leads to thrombosis of the submucosal veins. The numerous thromboses inhibit further iron absorption so that comparatively little iron is found in the liver and kidneys. It is difficult to come to another conclusion than that the extensive fatty changes in parenchymatous organs (liver heart, kidney) cause the rapid fatal outcome. The liver is not able to maintain its detoxicating function the heart becomes hemodynamically insufficient because of extensive fatty degeneration of its muscle fibers and the kidneys are damaged by the low blood pressure and fatty degeneration of tubular cells.

The cause of sudden death following relative wellbeing was obviously due to fatty degeneration of parenchymatous organs. Hyper ventilation and pronounced acidosis, suggesting a disturbance in oxydative metabolism, was present. In a recent investigation WITZLEBEN and CHAFFEY [11] found extensive hepatic necrosis following

Prostatahypertrophie festgestellt wurde. Diese wurde zunächst konservativ behandelt; als es im April 1965 jedoch zu einer akuten Harnverhaltung kam, wurde eine Ausschälung des Adenoms vorgenommen. Die histologische Untersuchung des Operationspräparates ergab keinen Hinweis auf Malignität. Nach der Operation war der Patient beschwerdefrei. Im Mai 1966 traten plötzlich am ganzen Körper blaue Flecken auf, außerdem bemerkte der Patient eine ausgedehnte Blutung unter der Bindehaut. Einige Tage später kam es außerdem zu einer Hämaturie, die so stark wurde, daß der Patient schließlich auf eine urologische Abteilung aufgenommen werden mußte. Hier ergab die i.v. Pyelographie und die zystoskopische Untersuchung keinen Hinweis auf eine lokale Blutungsursache. Aus diesem Grund wurde nun eine Gerinnungsuntersuchung durchgeführt. Diese ergab eine schwere Gerinnungsstörung (siehe später), weshalb der Patient am 4. 7. 1966 zur weiteren Abklärung und Behandlung an die I. Medizinische Klinik verlegt wurde.

Die klinische Untersuchung bei der Aufnahme an der I. Medizinischen Klinik ergab folgenden Befund: Reduzierter Allgemeinzustand, blasse Hautfarbe, an der Haut keine Zeichen einer hämorrhagischen Diathese. Herz und Lunge physikalisch unauffällig. Leber nicht vergrößert, Milz 1 QF unter dem Rippenbogen tastbar, keine Lymphknotenvergrößerung, Nierenlager nicht klopfempfindlich. Rektale Untersuchung: kein abnormer Befund an der Prostata erhebbar. RR 170/90 mm Hg. Es bestand eine leichte Hämaturie.

Laboratoriumsbefunde: Senkung 70/46 mm, Serumeiweiß 6,7 g%, Albumin 3,1 g%, Globulin 3,6 g%. Thymoltrübungsreaktion 1 E; Elektrophorese: Alpha$_1$- und Alpha$_2$-Globulinvermehrung. LAP 11 7 mE, Serumbilirubin 0,42 mg%, Bromsulphaleinretention nach 45 min 2%, Cholesterin 240 mg%, Harnstoff-Stickstoff 31 1 mg%, alkalische Phosphatase 3,1 m Mol E, saure Phosphatase 1,2 m Mol E. Blutbefund: Ery 3,9 Mill, Hb 75%, FI 0,96. Leukozyten 6.550, Unsegmentierte 5%, Segmentierte 74%, Eosinophile 2%, Basophile 2%, Monozyten 1%, Lymphozyten 16%. Harnbefund: Albumen positiv, Saccharum negativ, Urobilinogen 1 4, Sulkowitsch positiv. Im Sediment reichlich Erythrozyten.

Röntgenuntersuchungen: Thoraxröntgen: Linksherz mit beginnender Mitralisation, vermehrte Grundhelligkeit der Lungen. Kein Hinweis für blastomatöse Veränderungen. Magen-Darm-Passage: normaler Befund. i.v. Pyelographie: Dürftige Ausscheidung des Kontrastmittels in beiden Nieren mit vor allem rechts unvollständiger Darstellung des Hohlsystems. Unbehinderter Abfluss des Kontrastmittels in die nicht vollkommen zur Darstellung gebrachte Harnblase. Verdacht auf Anhebung des Blasenbodens bei Vergrösserung der Prostata. Skelettröntgen: Hochgradige Spondylose mit multiplen Bandscheibendegenerationen und Osteochondrose im Bereiche der Brustwirbelsäule. Mäßige Osteoporose.

Gerinnungsuntersuchungen

Bei der ersten Untersuchung des Patienten konnten folgende Befunde erhoben werden (Tab. 1).

Als wesentliche pathologische Befunde wurde eine schwere Fibrinogenopenie bei gleichzeitiger Thrombopenie gefunden. Ferner bestand eine leichte Verminderung des Prothrombins und von Faktor V. Es war auch eine leichte Verminderung von Faktor VIII anzunehmen, da der Thromboplastinbildungstest unter Verwendung von Patientenplasma leicht pathologisch war. Eine vermehrte Fibrinolyse konnte mit dem Fibrinplattentest nicht nachgewiesen werden, die Euglobulinlysezeit war bei der ersten Untersuchung normal, bei späteren Untersuchungen konnte jedoch gelegentlich eine verkürzte Euglobulinlysezeit gefunden werden (Abb. 2).

Das gleichzeitige Vorkommen einer Fibrinogenopenie und einer Thrombopenie deutete mit grosser Wahrscheinlichkeit auf eine Verbrauchskoagulopathie. Zur Verifizierung dieser Diagnose wurde hier noch die biologische Halbwertszeit von jodmarkiertem humanem Fibrinogen bestimmt. Wie zu erwarten, war die Halbwertszeit des Fibrinogens bei dem Patienten mit 12 h deutlich verkürzt (Abb. 1). Die Halbwertszeit wurde noch kürzer (9 h)

Tabelle 1 Gerinnungsbefunde 24. 6. 1966

	Pat. H. L.	Normalwerte
Gerinnungszeit (LEE WHITE) min	6	9
Blutungszeit (DUKE) min	7[**]	– 3
Thrombocytenzahl (BRECHER und CRONKITE)	80 000	150 000 250 000
Thromboplastinzeit (QUICK), %	68	75–110
Prothrombin (F II) (DEUTSCH und SCHADEN) %	65	75–110
Proaccelerin (F V) (DEUTSCH und Schaden), %	67	75–110
Fibrinogen (CLAUSS) mg%	40	200–400
Partielle Thromboplastinzeit (LANGDELL) sec	77	50–70
Thromboplastinbildungstest (BIGGS)		
Pat.-Pl-Pat.-Serum	normal	
Pat.-Pl-Normalserum	leicht path.	
Serumprothrombin nach 1 h, %	50	< 10
Euglobulinlysiszeit h	über 8	über 12
Fibrinplatte	keine fibrinolytische Aktivität	

ls die Fibrinolyse durch tägliche orale Verabreichung von 10 g Epsilon-Aminocapronsäure gehemmt wurde. Wurde hingegen eine Dauerinfusion von Heparin verabreicht, kam es sofort zu einer deutlichen Verlängerung der Fibrinogenhalbwertszeit, wobei allerdings der Normalwert nicht erreicht wurde. Damit war erwiesen, dass die beobachteten Gerinnungsveränderungen, vor allem die Fibrinogenopenie und Thrombopenie, durch eine intravaskuläre Gerinnung bedingt waren.

Beeinflussung der hämorrhagischen Diathese durch Heparin und weiterer Verlauf der Krankheit

Bei der Annahme einer Verbrauchskoagulopathie infolge intravaskulärer Gerinnung war zu erwarten, dass eine Unterbrechung des intravaskulären Gerinnungsvorganges durch Heparin die Gerinnungsstörung beseitigen würde. Wie aus Abb. 2 zu entnehmen ist, kam es tatsächlich schon 12 h nach Beginn der Heparintherapie (1000 E Heparin/Stunde als Dauerinfusion) zu einem deutlichen Anstieg des Fibrinogens, das am 4. Tag nach Therapiebeginn einen Höchstwert von 410 mg% erreichte. Die Thrombocyten stiegen ebenfalls an, aber erst nach einigen Tagen und nicht in demselben Ausmass wie das Fibrinogen. Gleichzeitig mit dem Anstieg des Fibrinogens und der Thrombocyten kam es zu einem Sistieren der Blutungsneigung.

Der gute therapeutische Erfolg konnte aber nur so lange aufrechterhalten werden, als Heparin kontinuierlich infundiert wurde. Als aus technischen Gründen die Therapie mit Depotheparin subkutan fortgeführt wurde, kam es wiederum zu einem Abfall des Fibrinogens, wenn auch nicht auf so tiefe Werte wie vor der Therapie.

Der weitere klinische Verlauf war gekennzeichnet durch einen rasch zunehmenden Kräfteverfall und Gewichtsverlust. Bei wiederholten rektalen Untersuchungen konnte schliesslich an der Prostata ein Tastbefund erhoben werden, der den Verdacht auf ein Prostatakarzinom nahelegte. Eine Probepunktion zur Verifizierung dieser Diagnose konnte

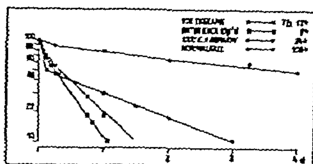


Abb. 1 Halbwertzeiten von J^{131}-Fibrinogen vor und während Therapie mit EACA 10 g tgl. bzw. Heparindauerinfusion (1000 E/h). Abszisse: Zeit in Tagen. Ordinate: Radioaktivität in % des 10-Minutenwertes.

Abb. 2. Einfluß der Heparintherapie auf Fibrinogenspiegel und Thrombozytenzahl.
—— Fibrinogen in mg%
●——● Thrombozytenzahl

wegen der hämorrhagischen *Diathese* aber nicht *durchgeführt* werden. Die saure Phosphatase war bei wiederholten Kontrollen nur grenzwertig. Der Patient verstarb plötzlich unter den Zeichen einer zerebralen Blutung am 3.8.1966.

Pathologisch anatomischer Befund

Bei der Obduktion (Sekt. Prot. Nr. 1145/66, Obd. REGELE, Auszug) des Patienten fand sich ein Prostatakarzinom mit Befall der regionären und paraaortalen Lymphknoten und an-



Abb. 3. Fibrinthrombus in einem kleinen Lungengefäß. Mallory (× 300).

gedehnten Knochenmetastasen in der Wirbelsäule. In Leber und Lunge waren makroskopisch keine Metastasen nachweisbar. Als unmittelbare Todesursache war eine frische Massenblutung in die rechte Kleinhirnhemisphäre anzusehen. Weitere Zeichen einer schweren hämorrhagischen Diathese lagen in Form massiver Muskelblutungen im rechten Oberschenkel und verschiedenen subkutanen Hämatomen vornehmlich im Bereiche von Injektionsstellen vor.

Histologisch erwies sich das Prostatakarzinom als ein Adenokarzinom mit wechselndem Gehalt an doppelt-brechenden sudanophilen Tröpfchen im Plasma. Retroperitoneale Lymphknötchen waren ausgedehnt von analogen Karzinomformationen durchsetzt, Nekrosen oder Fibrinabscheidungen fehlten dabei. In den makroskopisch tumorfreien Lungen war im histologischen Schnitt eine geringgradige Lymphangiosis carcinomatosa, vor allem aber auch Tumorformationen in kleinsten Gefäßen und interalveolären Kapillaren zu sehen, vereinzelt und dabei unabhängig von den Tumorzellen gelegentlich auch Fibrinthromben (Abb. 3). Das Myocard ohne Auffälligkeiten, insbesondere die Gefäße mit freier Lichtung. Auch die Leber zeigte keine gröberen Veränderungen, in den Sinusoiden keine Fibrinthromben. Die Kupfferschen Sternzellen relativ gross und mit stark eosinophilem Plasma. Dieses zeigte bei der PAS-Reaktion eine deutliche Rotfärbung, die auch nach Diastasebehandlung bestehen blieb. Eisenfärbung negativ.

Der für diesen Fall wesentliche Befund war in den Nieren zu erheben. So gut wie alle Glomerula zeigten homogene, eosinophile Ausgüsse in ihren Kapillarschlingen (Abb. 4). Diese gaben positive Fibrinfärbung und waren stets von der Kapillarwand abgrenzbar. Sie waren demnach als hyaline oder Fibrinthromben anzusprechen. Schlingennekrosen fanden sich nicht. Grössere und kleinere Rinden- und Markgefässe waren frei von Thromben.

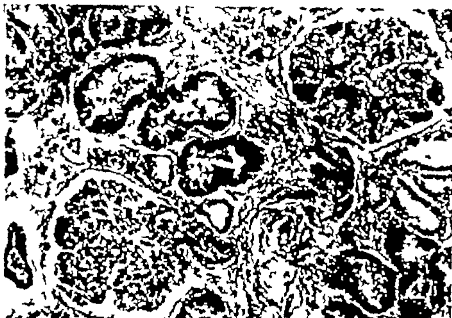


Abb. 4 a Hyaline Thromben in Schlingen von Nierenglomeruli. Mallory (× 400).

Diskussion

Die grosse Häufigkeit thromboembolischer Ereignisse bei Patienten mit malignen Tumoren ist dem Kliniker und Pathologen wohlbekannt. Eine wesentliche Ursache der Thromboseneigung beim Tumorkranken stellt sicherlich die Hyperkoagulabilität dar, die vor allem durch Vermehrung oder Aktivitätssteigerung verschiedener Prokoagulantien [13] (Fibrinogen, Faktor V und Faktor VIII) zustande kommt.

Klinisch äussert sich diese Thromboseneigung zumeist in Form von Makrothrombosen oder der sogenannten Thrombophlebitis migrans. Bei einer kleinen Zahl von Fällen führt die Hyperkoagulabilität aus bisher noch nicht genau bekannten Gründen jedoch zu einem anderen klinischen und pathologisch-anatomischen Bild. Es kommt zur Bildung zahlreicher Fibrinthromben in den Kapillaren verschiedener Organe, ein Zustandsbild, das als diffuse intravaskuläre Gerinnung bezeichnet wird. Dadurch kommt es zum raschen Verbrauch von Gerinnungsfaktoren, vor allem des Fibrinogens und der Thrombozyten, so dass eine Gerinnungsstörung entsteht, die vor allem durch eine Fibrino-

genopenie und Thrombozytopenie gekennzeichnet ist und nach ihrer Entstehung als Verbrauchskoagulopathie [6] bezeichnet wird.

Der vorliegende Fall weist alle charakteristischen Befunde einer solchen Verbrauchskoagulopathie auf: Fibrinogenopenie, Thrombozytopenie, Verminderung von Faktor V und von Prothrombin. Auch die zeitweise nachgewiesene Fibrinolyse gehört zum Bild der Verbrauchskoagulopathie, da der Körper versucht, die Fibrinthromben durch Aktivierung der körpereigenen Fibrinolyse wieder zu beseitigen. Erwartungsgemäss war die Halbwertszeit des Fibrinogens deutlich verkürzt. Durch Gabe von Heparin konnte die Fibrinogenhalbwertszeit zumindest teilweise normalisiert werden, was als Beweis dafür anzusehen ist, dass die intravaskuläre Gerinnung tatsächlich pathogenetisch im Vordergrund stand. Gleichzeitig führte die Heparinbehandlung, solange sie intensiv durchgeführt wurde, zur Beseitigung der ursprünglich bestehenden Gerinnungsstörung und der Blutungsneigung.

Auch das morphologische Substrat dieses Zustandsbildes konnte in diesem Fall eindeutig nachgewiesen werden. Es fanden sich Fibrinthromben in grosser Zahl in den Kapillaren der Niere und im geringeren Ausmass auch in der Lunge. Der Nachweis von Fibrinthromben bei der Autopsie gelingt auch bei sicheren Fällen von Verbrauchskoagulopathie nur selten, was wahrscheinlich darauf zurückzuführen ist, dass die aktivierte Fibrinolyse und die postmortale Autolyse die vorhandenen Fibrinthromben rasch weiter verändern.

Verbrauchskoagulopathien können prinzipiell bei jedem malignen Tumor [4, 14, 17] auftreten. Allerdings neigen bestimmte Karzinome, wie die Karzinome des Gastrointestinaltraktes [1, 3, 5, 8], das Prostatakarzinom [5, 15, 16, 18, 19, 21] und das Pankreaskarzinom [5, 9] besonders häufig zu dieser Komplikation. Das Prostatakarzinom weist insofern eine Sonderstellung auf, als hier eine Defibrinierung nicht nur durch eine intravaskuläre Gerinnung, sondern auch durch eine Hyperfibrinolyse [10, 20] zustande kommen kann, was durch den reichlichen Gehalt des Prostatagewebes an fibrinolytischen Fermenten erklärlich ist. Es scheint aber, dass bei allen Fällen von Defibrinierungssyndrom beim Prostatakarzinom prinzipiell immer beide Vorgänge – nämlich Verbrauch und Hyperfibrinolyse – eine Rolle spielen, wobei einer der Mechanismen pathogenetisch im Vordergrund steht.

Wieso es gerade in bestimmten Karzinomfällen zur Verbrauchskoagulopathie kommt, ist noch nicht ausreichend geklärt. Es fällt aber auf, dass diese Fälle gewisse pathologisch-anatomische und

klinische Gemeinsamkeiten haben. Pathologisch-anatomisch handelt es sich zumeist um Adenokarzinome. Es war in den bisher beschriebenen Fällen immer eine hämatogene Metastasierung nachzuweisen, wobei sich Metastasen stets in Knochen oder Lunge oder in beiden fanden. Lebermetastasen wurden hingegen so gut wie immer vermißt. Klinisch waren diese Fälle im allgemeinen durch einen besonders foudroyanten Verlauf charakterisiert, der wahrscheinlich als Ausdruck des Einsetzens einer massiven hämatogenen Metastasierung anzusehen ist. Innerhalb kurzer Zeit könnten aus den zirkulierenden Tumorzellen beträchtliche Mengen an thromboplastischen Substanzen freigesetzt werden, wodurch die Clearancekapazität des RES und die körpereigenen Inhibitoren überspielt werden. Damit wären die Voraussetzungen für eine intravaskuläre Gerinnung gegeben.

Zusammenfassung

Es wird der Fall eines 71jährigen Patienten mit einem Prostatakarzinom beschrieben, bei dem es akut zum Auftreten einer Blutungsneigung kam. Als Ursache wurde eine Verbrauchskoagulopathie festgestellt. Durch Gabe von Heparin konnten die Gerinnungsstörung zeitweise beseitigt und die ursprünglich verkürzte Fibrinogenhalbwertszeit verlängert werden. Bei der Autopsie wurde ein Prostatakarzinom mit regionären Lymphknotenmetastasen und hämatogenen Metastasen in den Knochen und der Lunge gefunden. Histologisch konnten in der Niere und der Lunge Fibrinthromben nachgewiesen werden.

Summary

A 71 years-old patient with metastatic prostatic cancer suddenly developed severe hemorrhagic diathesis. Fibrinogenopenia, thrombocytopenia, reduction of the factors II, V and VIII and a shortened half-life time of ^{131}J labeled fibrinogen were found. Diffuse intravascular clotting was suggested and heparin by continuous infusion was administered. This led to marked increase of fibrinogen and partial correction of the shortened half-life time of fibrinogen. At autopsy adenocarcinoma of the prostata with bone and lung metastases was found. Fibrin thrombi could be detected in the capillaries of the kidney and the lung.

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A Rapid Clinical Method for the Estimation of Activity of Aconitase in Blood

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The enzyme aconitase belongs to the class of hydrase enzymes which add water to organic compounds without splitting The reaction may involve an intermolecular oxydation reduction coupled with final hydration process. This is often a preliminary reaction step before starting biological oxydation

The methods for the estimation of aconitase are based on the fact that it converts citrate to cis-aconitate which is further transformed into *dl* isocitrate Citrate $\rightleftharpoons$ cis-aconitate $\rightleftharpoons$ *dl* isocitrate.

In a simple spectrophotometric method, the light absorption due to cis-aconitate at 240 nm wave length is measured as an index of the enzyme activity in presence of citrate or cis-aconitate as the substrate [1] This procedure is unsatisfactory for direct estimation of enzyme activity in presence of haemoglobin or blood protein which has a high extinction coefficient at 240 nm wave length. The enzyme activity may also be measured from the amount of citric acid produced from cis-aconitic acid by the enzymatic reaction and determined colorimetrically as a pentabromoacetone derivative [2] This method is accurate but not suitable for rapid clinical assay of blood aconitase

While exploring the possibility of obtaining a reliable method suitable for rapid evaluation of the status of aconitase in human blood, a simple method was evolved The principle of this method is as follows The activity of the enzyme is determined colorimetrically by following the rate of reduction of the redox dye 2–6-dichlorophenol indophenol in presence of sodium citrate, pig heart extract (isocitric dehydrogenase) nicotine adenine dinucleotide phosphate (NADP)

manganese chloride and phosphate buffer pH 7 4 The rate of reduction is followed in a Leitz photoelectric colorimeter at 610 nm wave length. A suitable substrate blank without citrate and NADP is run simultaneously to eliminate non-specific reduction of the dye This method is modified from the spectrophotometric method employed by Barban and Schulze [3] The unit of activity is taken as the rate of change of optical density per minute under the condition of the test.

Material and Method

Five ml of venous blood collected using heparin as the anticoagulant are used for colorimetric determination of activity of aconitase as detailed below The same sample is also analysed by standard spectrophotometric [4] method for the estimation of aconitase activity

Colorimetric method

Immediately after collection, the blood is transferred to an ice bath; 0.5 ml of whole blood is dissolved in 4.0 ml of ice cold distilled water and kept in the ice bath for 10 to 15 min when the sample ppears clear Then 0.5 ml of 0.2 M phosphate buffer pH 6.8, is mixed with the solution and preserved in the ice bath till the end of the assay

Into pyrex test tube (6″ × ¾″), the following reagents are added and maintained at 38° in water bath Phosphate buffer pH 7 4, 0.2 M 1.0 ml 0.06 M Sodium citrate 0.5 ml; distilled water 3.9 ml manganese chloride 0.001 M 0.1 ml.

A blank reaction mixture tube is also prepared as follows Phosphate buffer pH 7 4 0.2 M 1.0 ml distilled water 4.4 ml manganese chloride 0.001 M 0.1 ml.

Pig heart acetone powder extract in 0.1 M phosphate buffer of pH 7 4 containing 50–100 μg of protein/ml prepared and preserved at 4° C is used as source of isocitric dehydrogenase.

A NADP solution containing 2 mg of NADP (Sigma) per ml of distilled water is placed in test tube in ice bath.

A Leitz photoelectric colorimeter is adjusted to 100% transmission against distilled water at 610 nm wave length 0.1 ml of 2–6-dichlorophenol indophenol (0.035%) is taken in 1 cm² cuvette. Pig heart extract 0 1 ml, NADP solution 0.1 ml and 0.2 ml of blood solution (1 in 10 dilution) are added in succession to reaction mixture tube maintained at 38 °C in water bath. The whole mixture is then poured into the cuvette containing the dye. This procedure ensures thorough mixing of the dye and the reactants. A stop watch is started after taking the initial (zero time) reading. Subsequent readings are taken at an interval of 1 min for 4 min. The above procedure is repeated with blank reaction mixture

The rate of change of optical density (O.D) is calculated for both the substrate and blank reaction mixtures from the linear part of the activity curve. The corrected value 'delta (Δ) is obtained by subtraction of the value for blank activity from that of substrate activity

All estimations are done in duplicate and the unit of enzyme activity is taken as the rate of change of O.D. per minute under the condition of the test.

The final enzyme activity is calculated as follows

Corrected 'delta	= 0.0047
PVC (packed cell volume)	= 32
Dilution factor	$= \frac{6 \times 10}{0.2} = 300$

Table III. Units of aconitase activity in iron deficiency anaemia

Name	Hb g%	PCV %	Ret. %	WBC/mm³	Enzyme activity/ ml blood/min
S. D	6.09	25	7.4	7,700	0.35
G. R.	5.8	22	6.6	3,300	1.12
D. N.	5.2	19	4.0	8,200	0.35
J. N. D.	5.2	23	0.9	7,100	0.79
R. B.	8.7	30	1.9	6,300	0.65
A. P	9.8	31	1.1	18,400	1.13
S. B.	5.5	27	1.3	6,800	1.27
P. C.	6.58	26	1.8	7,500	0
B. J	6.58	24	5.5	11,900	0.65
H. S.	3.19	16	12.0	5,600	0.48

Values for activity of aconitase obtained *colorimetrically* in *normal* and iron deficiency subjects are shown in tables II and III

Comment

In the system employed the redox dye 2.6-dichlorophenol indophenol probably plays the following role Citrate → cis-aconitate → *dl*-isocitrate *Dl* isocitrate + NADP → oxalo-succinate + NADPH NADPH + oxydized dye → NADP + reduced dye. The reduced dye does not undergo spontaneous oxydation and the fall in optical density is used as a measure of the enzyme activity The dye 2.6-dichlorophenol indophenol has also been employed in enzymatic assay of oxydized and reduced forms of NADP and diaphorase in colorimetric studies [5 6] The reducing effects of the substrate blank solution is due to non specific reducing substances likely to be present in blood lysates. The linearity curve shown in figure 1 is plotted after subtracting the blank value. Slope of the line or Δ expressed as the unit of enzyme activity per minute, by both spectrophotometric and colorimetric methods, gave comparable results as shown in table I It would appear that for rapid clinical estimations, colorimetric method may be substituted for spectrophotometric method. This method is sensitive enough to detect differences in the enzyme activity of normal and anaemic subjects particularly those with iron deficiency anaemia [7]

Acknowledgement

Grateful thanks are due to K. K. MATHEN, All India Institute of Hygiene and Public Health, for statistical help.

Summary

A simple colorimetric method has been standardised for the rapid determination of aconitase activity in whole blood by using redox dye 2-6-dichlorophenol indophenol in the presence nicotine adenine dinucleotide phosphate isocitric dehydrogenase citrate and phosphate buffer pH 7.4. Comparison of the data on the same sample obtained colorimetrically and spectrophotometrically showed no statistically significant difference. Evaluation of the enzyme activity in blood of normal and iron deficient subjects showed a deficiency of aconitase in the latter group.

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R. Dybkaer und K. Jørgensen: Quantities and Units in Clinical Chemistry. Munksgaard, Copenhagen 1967

In den letzten Jahren hat sich die klinische Chemie immer mehr zu einem selbständigen Fach entwickelt. Eine Vielzahl von körpereigenen Stoffen werden mit immer komplizierteren Apparaten unter teils komplexen Versuchsbedingungen analysiert. Ein echter Fortschritt ist aber nur dann gewährleistet, wenn eine auf der ganzen Welt einheitliche Terminologie in bezug auf die analysierten Stoffe und Ausdrucksformen in bezug auf Grössen und Einheiten verwendet wird. Welche praktisch medizinischen Fortschritt die Wahl einer richtigen Einheit mit sich bringen kann, hat der Übergang von g/100 ml auf mAeq/Liter bei den Plasma-Elektrolyten gezeigt. Ähnliches muss auch auf allen anderen Bereichen angestrebt werden. Das Buch von Dybkaer und Jørgensen liefert nicht nur Ansätze zu einer solchen einheitlichen Terminologie der Mass-Einheiten, sondern legt ein weitgehend vollständig ausgearbeitetes System vor. Das Buch sollte von jedem gelesen werden, der sich mit wissenschaftlichen Fragen jeder Art auseinandersetzt. Nur dann ist zu hoffen, dass unverständliche und zweideutige Mass-Einheiten wie g% aus der Literatur verschwinden.

R. Richterich, *Bern*

R. Waitz: Actualités en transfusion sanguine. 2e cours européen de transfusion sanguine organisé par le Conseil de l'Europe Strasbourg 1966. Masson, Paris 1967. 184 S., 24 Abb., 50 Tab. Preis 46 F

Seit einiger Zeit organisiert der Europarat alljährlich einen Kurs für Transfusionskunde, zu welchem die Mitgliederländer des Rates jeweils 1–2 jüngere Spezialisten dieses Fachgebietes delegieren. Der zweite Kurs fand vom 3.–14. Mai 1966 unter Leitung von Prof. R. Waitz im Blutspendezentrum Strassburg statt. Die praktischen Kursarbeiten wurden umrahmt von 11 Übersichtsreferaten, gehalten von den führenden Fachleuten Frankreichs. Der vorliegende Band vereinigt diese Referate und vermittelt dadurch einen ausgezeichneten Überblick, namentlich über die immunhämatologischen Aspekte der Bluttransfusion.

A. Hässig, *Bern*

Primates in Medicine. Vol. 1: First Holloman Symposium on Primate Immunology and Molecular Genetics. Ed. by C. H. Kratochvil. Karger, Basel/New York 1967. XVI + 99 p., 53 fig., 28 tab. Price sF /DM 16.50, US $ 4.00/30 s.

The book contains contributions from eight research groups which were presented at the Symposium held on September 28/30, 1966, at the Aeromedical Research Laboratory of the *Holloman Air Force Base, New Mexico* (USA). Simultaneously with the increase of knowledge on human immuno-genetic systems, the study of such systems in non-human primates is gaining growing interest. Six out of the eight studies are dealing with this subject. Moor-Jankowski and Wiener are giving a survey on human-type simian-type and crossimmune-type of blood groups in primates. Masouredis *et al.* are presenting new data on the nature of Rh-antigens, obtained with the method of I 125 and Rh_0 (D) uptake on the red cells. Wiener *et al.* are reporting on the complex relationship of the chimpanzee C-c E-F system to the human Rh-Hr system. Porter's paper is dealing with the structural similarities between immunoglobulins of human, chimpanzee and other non-human primates and Alepa is presenting evidence that chimpanzee sera contain at least four Gm- and two Inv-factors, known to be isoantigenic determinants of human γG-immunoglobulins. Finally Goodman reports on his immunological investigations on proteins of primates

which – besides other interesting results – have revealed extensive transferrin polymorphisms in *Macaca mulatta*, chimpanzees and gorillas. Out of the two remaining studies, one by Hoffman and Gottlieb deals with hemoglobin variants of chimpanzees and gibbons, the other by Kendall, is an investigation on non-toxic immune-suppressive agents (performed not on primates but on mice). The Symposium has provided valuable informations on current researches in primate immunology and molecular genetics. Each of the studies gives a lot of ideas and suggestions for further investigations which will also interest scientists working exclusively in human serology.

R. Bütler, *Bern*

J. Gancz: Das «Banti-Syndrom» im Kindesalter. VIII+133 S., 31 Abb., 14 Tab. Enke, Stuttgart 1968. *Preis* DM 36.–

Der Autor bemüht sich um eine Klärung des Begriffes «Banti-Syndrom». Neben der Aufzählung symptomatischer Formen, insbesondere bei Pfortaderhypertonie, hält er an der Existenz einer primären, essentiellen Form fest. Er beschreibt im Detail die klinischen Symptome und den Verlauf bei 15 Patienten, die er hier einreiht. Durch Bestimmen erythrozytärer, leukozytärer und thrombozytärer Antikörper wird versucht, einen Beitrag zur Ätiologie des Syndroms zu liefern. Auch die Rolle der Milz im Sinne einer Dysplenie und die Zellphagozytose werden erörtert. Die spätere Mitbeteiligung der Leber im Sinn einer Zirrhose wird ebenfalls z.T. auf Autoaggression zurückgeführt. Trotz den vielfältigen Untersuchungen bleiben viele Theorien in dieser anregenden Betrachtung eines schwierigen Problems spekulativ.

M. Vest, Basel

J. M. Collette, G. Jantet and E. Schoffeniels: New Trends in Basic Lymphology. Proceedings of a Symposium held at Charleroi (Belgium) on 11 to 13 July 1966. Experientia, Supplementum 14. Birkhäuser, Basel 1967. 233 S. Preis F. 48.–

The papers on the rather young specialty of lymphology presented in the Symposium at Charleroi are reproduced in extenso in this volume. They concern the structure and ultrastructure of the lymphatic vessels, the properties and permeabilities of the lymphatic endothelium, the contractility of human lymphatics, the problem of lymphaticovenous communications, the effects of lymphatic stasis and the various methods for exploration of lymphatic structure and function.

There is no question that such a booklet serves as a valuable source of information to the basic scientist as well as the clinical investigator interested in lymphology.

P. Frick, *Zürich*

Proceedings of the 10th Congress of the European Society of Haematology, Strasbourg 1965. Part I: Hauptvorträge. 312 Seiten. *Preis* sFr./DM 75.–. Part II: *Kurzvorträge* (2 Teile, 814 und 774 Seiten). Preis für beide Teile: sFr./DM 370.–

Traditionsgemäss erscheinen die Beiträge des 10. Kongresses der Europäischen Gesellschaft für Hämatologie in sorgfältig ausgestatteter Buchform. Der erste, bereits vor Jahresfrist veröffentlichte Teil enthält die Hauptreferate. Der Band ist für jeden hämatologisch orientierten Arzt und Biochemiker ein wertvolles Quellenwerk, welches auf knappem Raum neuere Aspekte in sorgfältig redigierten Übersichten über zentrale Probleme der Hämatologie zusammenfasst. Die Arbeiten von Sadron über Methoden der Untersuchung von Makromolekülen in Lösung und diejenige von Kabat über Struktur und Heterogenität von Antikörpern zeigen die Bedeutung und Fruchtbarkeit interdisziplinärer Zusammenarbeit zwischen Kliniker und Biochemiker. Weitere, durchwegs originell dargestellte Beiträge behandeln die Geographie hämatologischer Erkrankungen, Probleme der Blutgerinnung, Fibrinolyse und Serologie, sowie neuere Aspekte der Leukämieforschung. Von besonderem praktischem Interesse ist die Arbeit von Lawkowicz und Czerski über die vergleichende Hämatologie verschiedener Tiergattungen, welche häufig für die experimentelle Forschung herangezogen werden.

or chronic renal insufficiency can be associated with a haemorrhagic disorder. In the same year SALZMAN and NERI [5] and CASTALDI *et al.* [6] investigated the dynamic platelet functions in the haemorrhagic diathesis of uraemia. This paper, which is a continuation of that presented in 1966, describes the haemostatic mechanism in 19 cases of uraemia and deals mainly with platelet adhesiveness.

Material and Methods

Patients. The haemostatic mechanism was investigated in 19 uraemic patients who showed signs of a haemorrhagic disorder (tendency to epistaxis, spontaneous haematomas) at some stage of the disease. At the time of the examination 5 of these patients showed *cutaneo-mucosal* haemorrhages (purpura, haematomas, haemoptysis). All these patients had severe renal impairment with high blood urea levels (over 150 mg/100 ml), markedly reduced glomerular filtration rate and isothenuria. A few of them were treated by peritoneal dialysis and the others were on the waiting list for renal transplantation.

Methods. The platelet function was investigated using the following tests:

1. Bleeding time. OWREN's method [7] was used. In our hands the normal values were between 5 and 7 min. The bleeding time is determined by the vasoconstriction, platelet adhesiveness, aggregation and viscous metamorphosis.

2. Platelet adhesiveness. We used BORCHGREVINK's test which measures platelet adhesiveness in vivo [8]. Two platelet counts are made: one in venous blood and another in the blood flowing from a 1 cm long and 1 mm deep skin incision in the forearm (the same incision was also used for the bleeding time test by OWREN's method). The difference between the two counts indicates the number of platelets adherent to the margins of the incision. The normal values represented as a percentage of the venous blood platelet count were, in our experience, between 20% and 48%, with an average value of 34% [9].

3. The difference between the maximal amplitudes (ma) of the thromboelastographic curves in platelet-rich and platelet-poor plasma, expressed as ma1–ma2. The ma1–ma2 value depends on the thrombodynamic function of the platelets which determines their participation in syneresis. For normal platelet counts the ma1–ma2 value varies between 20 mm and 25 mm.

4. Clot retraction. This test measures the retractive function of platelets.

5. Prothrombin consumption test. Normal results are more than 25 sec. Results below this limit are abnormal and, if one can be sure that there is no deficiency of any plasma factor which takes part in the generation of the intrinsic activator, they would indicate deficiency of platelet factor 3.

Results

Results are shown in table I. The prothrombin consumption test was abnormal in 17 out of 19 patients (89.4%) with an average time of 20.3 sec which is below the normal limit of 25 sec. This was, therefore, an almost constant finding. BORCHGREVINK's test showed decreased adhesiveness in 11 out of 19 patients (57.9%). The average value of 18.8% was much below the normal value of 34% ± 7%, and the differ

Table I. Chronic renal failure

Patients	No. of platelets per mm³ × 1000	Bleeding time min	TEG ma¹–ma² mm	Platelet adhesiveness in vivo %	Clot retraction	Prothrombin consumption index
1 F.L.	230	4	44	30	N	–
2 D. F	269	>20	39.5	20	N	21
3 A. A.	100	27	–	44	N	19
4 V. S.	298	15	29	8	N	–
5 D. C.	179	27	–	9	N	14
6 E. B.	90	–	–	22	N	19
7 P. S.	166	>20	45	0	N	15
8 J T	138	4	40	17	N	21
9 D. C.	118	3	40	13	N	16
10 A. S.	115	>20	42	11	N	15
11 J M.	381	–		22	N	–
12 S. P	310	5	–	0	N	31
13 L. B.	330	5	–	30	N	–
14 E. R.	340	15	34	15	N	30
15 J O	315	4	36	8	N	23
16 J. V.	283	3	26	43	N	21
17 O. D.	285	>15	46	36	N	19
18 C. C.	233	7	31	15	N	17
19 D L.	263	>13	34.5	17	N	24
Mean	236	>12	31.2	18.8		20.3

ence was statistically significant. This difference is well represented in figure 1 where a comparison is made between normal adhesiveness and adhesiveness in uraemia. The bleeding time was prolonged (over 7 min) in 9 out of 17 cases (52.9%). The thromboelastographic index ma1–ma2, which represents the thrombodynamic function of the platelets, was abnormal in 2 cases. Clot retraction was always normal. The platelet count was within normal limits (average 236.000/mm against the normal average of 262.000/mm³) except in one case where it was below 100.000/mm³

Discussion

The above reported results show impairment of, at least, two platelet functions (1) deficiency in the plasma of platelet factor 3 and (2) deterioration in the dynamic function of platelet adhesiveness. In addition, Castaldi has demonstrated impairment platelet aggrega

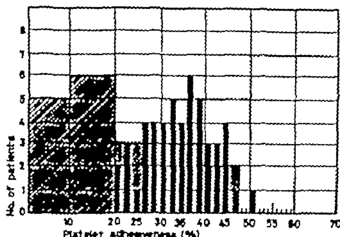


Fig 1 Comparison between the platelet adhesiveness in uraemia (obliquely striped zone) and that in 50 normal controls (solid columns)

tion. Thus, it is logical to accept that a true 'uraemic thrombopathy' does exist and is the cause of the haemorrhagic diathesis in chronic renal insufficiency. Platelet adhesiveness and aggregation become active during the first stage of haemostasis and their failure has a serious effect on early haemostasis. This is well demonstrated by the *rapid commencement of bleeding and its long duration when the* bleeding time test is performed. Although the bleeding time is not always prolonged, there is another characteristic feature of the abnormal test which is always present in cases of diminished adhesiveness. In normal subjects there is a time interval of 30–45 sec between the time the incision is made and the time when bleeding begins and forms *a drop of blood sufficiently large to be drawn into a pipette. In cases* of diminished adhesiveness this time interval is reduced to 5–10 sec. The possible cause of this phenomenon could be either absence of adhesive platelets to occlude bleeding vessels mechanically or the fact that abnormal platelets do not carry serotonin which contributes to the early vasoconstriction. In view of this observation, the axonal reflex postulated as the earliest haemostatic response *appears to be* much less important than dynamic platelet functions and, especially the first of these in chronological order i.e. adhesiveness. On the other hand, deficient generation of intrinsic thromboplastin impairs coagulation of the plasma which, in normal conditions, follows and reinforces the early phase of haemostasis.

Analysis of the correlation between the platelet adhesiveness, prothrombin consumption, thrombodynamic function and bleeding time in uraemia shows that

(a) Out of 13 uraemic patients with an abnormal prothrombin consumption the platelet adhesiveness was decreased in 8 and normal in 5 Two other patients with a normal prothrombin consumption showed decreased adhesiveness. Thus, these two tests of platelet function agreed in 8 and disagreed in 7 cases. Figure 2 shows a slight correlation between deficient prothrombin consumption and decreased adhesiveness but the number of tests is too small to attempt to express it in mathematical terms as an index of correlation (r) or covariation (p) The average values for both prothrombin consumption (20.3 sec) and adhesiveness (18.8 %) are below the normal limit.

(b) Comparison of the platelet adhesiveness with the thrombodynamic function shows that (1) Out of 9 cases with diminished adhesiveness the thrombodynamic function was normal in 8 and abnormal in 1 (2) out of 4 cases where adhesiveness was within normal limits the thrombodynamic function was normal in 3 and abnormal in 1 Thus, there is a considerable difference in the way these two platelet

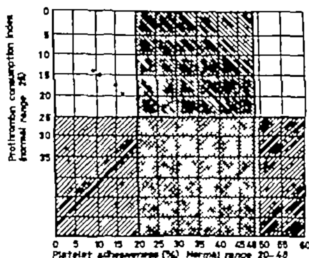


Fig 2 Correlation between platelet adhesiveness and the prothrombin consumption. The area divided into squares represents the normal range. Circles (O) indicate 'pairs of values' of adhesiveness and the prothrombin consumption. The cross (×) represents the average 'pair of values'

functions are affected adhesiveness is often impaired but the thrombodynamic function is usually normal.

(c) Finally the platelet adhesiveness and the bleeding time are compared in figure 3 which shows that (1) out of 11 cases of diminished adhesiveness the bleeding time was prolonged in 6 and normal in 5 (2) out of 6 cases with normal adhesiveness the bleeding time was normal in 3 and prolonged in the other 3 It is possible that decreased adhesiveness was compensated in some cases by vasoconstriction which participates in the haemostatic mechanism. The average adhesiveness was 18.8% and the average bleeding time 12 min. *There was therefore* some correlation between decreased adhesiveness and a prolonged bleeding time.

On the other hand, the syneresis and clot retraction which are related to the so-called thrombodynamic function of the platelets were usually normal obviously these platelet functions are independent of the others.

Very little is known about the pathogenesis of the platelet defect in uraemia. We can only report that decreased adhesiveness and aggregation are not apparently related to low haematocrit values so fre-

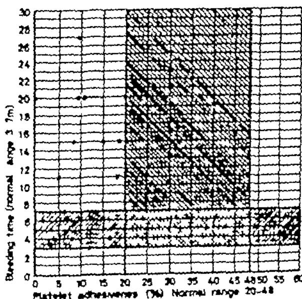


Fig. 3. Correlation between platelet adhesiveness and the bleeding time The area divided into squares represents the normal range Circles (○) indicate 'pairs of values of adhesiveness and the bleeding time. The cross (×) represents the average 'pair of values

quently found in uraemia [10 11] and that HELLEM [12] has suggested that urea may interfere with platelet aggregation induced by ADP the meticulous investigations of SALZMAN and NERI [5] refute this hypothesis

SALZMAN and NERI [5] and CASTALDI *et al* [6] using different techniques, obtained results similar to ours. SALZMAN investigated platelet adhesiveness *in vitro* (by this technique normal values are between 25 % and 60 %) in 24 patients and found marked reduction to between 0 % and 19% in 21 of them. He found impaired adhesiveness in 7 out of 8 cases of gastrointestinal haemorrhage associated with uraemia. Like us, he observed some correlation but not full agreement, between deficient adhesiveness and reduced prothrombin consumption. In his opinion, deficient adhesiveness is an important factor in the uraemic haemorrhagic disorder and in the prolonged bleeding time.

CASTALDI *et al* [6] investigated the haemostatic mechanism in 19 cases of renal disease, 12 of which had haemorrhagic manifestations in 5 of the latter they found platelet adhesiveness *in vivo* of less than 20 (the normal range with their technique was between 20 % and 70 %) The bleeding time by Ivy s method, was more than 15 min in all patients who were bleeding, and in only one who had no clinical signs of a haemorrhagic disorder Platelet aggregation was also abnormal in the cases with a haemorrhagic tendency

Thus, both our investigations and those of other authors show a defect in the adhesiveness, in addition to the well known diminished prothrombin consumption, which is probably related to the haemorrhagic tendency and prolonged bleeding time in uraemic nephropathy It seems, therefore, that a deficiency of platelet adhesiveness together with a deficiency of platelet factor 3 may possible be an important factor in the uraemic haemorrhagic diathesis. The thrombodynamic function, on the other hand, is usually normal.

Conclusions

In chronic renal insufficiency a decreased prothrombin consumption is the most frequent finding which indicates a deficiency of platelet factor 3 There is frequently diminished platelet adhesiveness which explains the prolonged bleeding time in some cases. The bleeding time is often prolonged. The thrombodynamic function is usually normal.

Summary

The bleeding time, platelet adhesiveness *in vivo*, thrombodynamic function of platelets, clot *retraction* and *prothrombin consumption* were investigated in 19 cases of chronic uraemia associated with a haemorrhagic diathesis at some stage *of the* disease. A decreased prothrombin consumption as a result of deficiency of platelet factor 3 was found in almost *all the cases, as well as very frequently a diminution in the platelet adhesiveness*, and often a prolonged bleeding time. It is suggested that impaired platelet adhesiveness and a deficiency of platelet factor 3 play an important role in the uraemic haemorrhagic disorder

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(Vorstand: Prof. Dr. H. B…)

Zur Diagnose monozytärer Leukämien mit zytochemischen Methoden[1]

F. Schmalzl und H. Braunsteiner

Neuere zytochemische Untersuchungen über die Herkunft der normalen Blutmonozyten haben die Theorie der myelogenen Abstammung dieser Zellen bestätigt [4, 14, 30, 31]. Mit der Klärung dieses Problems eng verbunden, ist die seit Schilling und Naegeli diskutierte Frage der Monozytenleukämien [3, 8, 22, 26, 28, 35]. Dem viel fach als «typische Monozytenleukämie» bezeichneten Typ Schilling, für den eine unmittelbare retikulohistiozytäre Abstammung der leukämischen Zellen bisher angenommen wurde, steht die sogenannte «myeloische Monozytenleukämie» Typ Naegeli gegenüber, deren Zellen von zahlreichen Autoren als Paramyeloblasten aufgefasst werden. Mischtypen [8] zwischen diesen Formen wurden beschrieben.

Diese Unterscheidungen wurden anhand panoptischer und phasenkontrastmikroskopischer Untersuchungsverfahren getroffen [3, 8, 22, 25, 28]. Mit der Anwendung zytochemischer Verfahren ist eine einfache und klare Differenzierung der Monozytenleukämie gegenüber anderen Hämoblastosen möglich geworden [4, 7, 11, 14, 17].

Material und Methodik

Der vorliegenden Arbeit liegen die Ergebnisse von Untersuchungen an 5 Fällen monozytärer Leukämien zugrunde. Es wurden Ausstriche von peripherem Blut und von Knochenmarkspunktionen verwendet. Die Resultate wurden zum Teil mehrfach kontrolliert.

[1] Diese Untersuchungen wurden mit Unterstützung des Fonds „Kampf dem Krebs“ durchgeführt.

Zur Durchführung gelangten folgende zytochemische Reaktionen

1. Unspezifische Esterase mit α-Naphthyl-Acetat als Substrat (α-N-E). Wir verwendeten hierbei die von Löffler angegebene Methode, die wir gering modifizierten [16, 30].

2. Unspezifische Esterase mit Substraten der Naphthol-AS-Reihe (N-AS-E). Es sind dies unterschiedlich substituierte Derivate des β-Naphthols, wie Naphthol-AS-, Naphthol-AS-D- und Naphthol-AS-LC-Acetat (N-AS-A). Diese Substrate geben übereinstimmende Resultate. Für den Nachweis der Hemmbarkeit der N-AS-E wurde Natriumfluorid (NaF) in der Konzentration von 1,5 mg/ml dem Puffer zugegeben [6, 16].

3. Naphthylamidase: Wir wählen diese unspezifische Bezeichnung, um jene Fermente zu kennzeichnen, die bei zytochemischen Nachweisverfahren Amide des β-Naphthylamins mit L-Leucin, D,L-Alanin oder D,L-Methionin spalten [1, 27, 29, 32] und die mit der echten Leucinaminopeptidase nicht identisch sind [5]. Geringe, offenbar substratbedingte Unterschiede im Verteilungsmuster des Enzyms lassen sich bei Verwendung der verschiedenen Substrate feststellen. Wir verwendeten für die vorliegenden Untersuchungen L-Leucyl-β-Naphthylamid. HCl mit der etwas modifizierten Technik nach Schwartz und Katzenstein [29, 32].

4. Die Darstellung der Naphthol-AS-D-Chloroacetatesterase (N-AS-D-Cl-E) erfolgte nach der von Moloney et al. [21] angegebenen Methode.

5. Saure Phosphatase wurde in Anlehnung an die Methode von Barka und Anderson [2] nachgewiesen.

6. Bei der Darstellung der alkalischen Phosphatase [9] und

7. der Myeloperoxydase [10] folgten wir den Angaben von Kaplow.

8. Die PAS-Färbung erfolgte nach Hotchkiss [23], die

9. Sudanschwarz B-Färbung nach Sheehan and Storey [33]. Kernfärbungen wurden entweder mit saurem Hämalaun nach Mayer oder mit der DNS-Färbung nach Feulgen durchgeführt.

Ergebnisse

Die Ergebnisse unserer ferment- und bausteinzytochemischen Untersuchungen an normalen Blut- und Knochenmarkausstrichen stimmen mit den Ergebnissen anderer Autoren weitgehend überein [7, 11, 14, 16, 17, 21, 30]. In der Tabelle I sind die Befunde der verschiedenen zytochemischen Reaktionen an leukämischen Monozyten zusammengefasst.

Unter den aufgeführten Fermentnachweisen zeigten insbesondere die sogenannte unspezifische Esterase und die Naphthylamidase auffällige Reaktionsergebnisse in den leukämischen Zellen (Tab. I).

Die Aktivität der unspezifischen Esterase war sowohl mit dem Substrat α-Naphthyl-Acetat wie auch mit den Substraten der Naphthol-AS-Reihe sehr hoch. Die hohe Aktivität der N-AS-E wurde durch NaF (1,5 mg/ml) in den normalen und leukämischen Monozyten vollständig gehemmt [6, 30].

Die Naphthylamidase, nachgewiesen mit den Substraten L-Leucyl-β-Naphthylamid HCl, liess in allen 5 untersuchten Leukämien eine deutliche Aktivität erkennen.

Naphthol-AS-D-Chloroacetat-Esterase war bei Fall 1 in mässigem Ausmass in den leukämischen Zellen nachweisbar. In den 4 anderen

Fällen fanden sich nur gelegentlich Spuren dieses Fermentes in den monozytären Zellen.

Sudanschwarz B-positive Phospholipoide liessen sich in allen 5 Fällen in geringem Ausmass feststellen. Peroxydase ebenfalls nur in geringem Ausmass, fand sich nur in einem Fall, die anderen enthielten kaum Peroxydaseaktivität in den leukämischen Zellen. Wir ziehen die Sudanschwarzfärbung der Peroxydasereaktion vor, da die Sudanschwarzfärbung in jüngeren, weniger ausdifferenzierten Zellen der myeloischen Reihe, die noch keine Peroxydase enthalten, bereits eine positive Reaktion gibt.

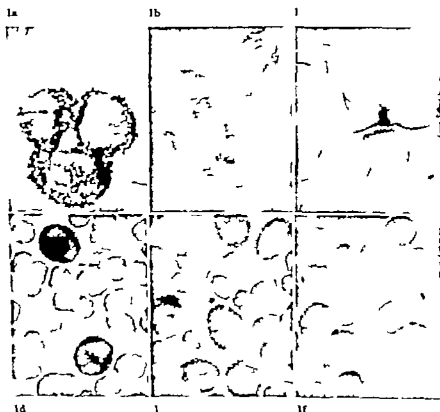


Abb. 1 Esteraseaktivität bei verschiedenen Leukämieformen. N-AS-E.) b) und) In) Fermentaktivität durch 1,5 mg/ml NaF gehemmt. α-N-E d)) und f). Stark positiv reife Monozytenleukämie) und d). Weniger stark positiv Promonozyten-Leukämie b)) N-AS-E durch NaF in den leukämischen Zellen gehemmt (selber Fall wie) f) deutliche α-N-E-Aktivität in Paramyeloblasten.) 1100 b)), d),) f) 1800 ×

Tabelle I Angaben über weisses Blutbild, alkalische Neutrophilenphosphatase und Verhalten in den untersuchten Fällen von Monocytenleukämie 0 keine ± unsichere,

	Leukocyten pro mm³	Monocytoide Zellen in %	Index der alkalischen Leukocytenphosphatase	α-Naphthyl-Acetat Esterase
Normale Blutmonocyten				++ bis +++
Fall 1 Pat. R. 25 J	3 400	48%	108	++ bis +++
Fall 2: Pat. L. 60 J	8 100	38%	40	++ bis +++
Fall 3 Pat. B. 39 J	12 000	56%	96	++ bis +++
Fall 4: Pat. W. 44 J	187 000	93%	307	+ bis +++
Fall 5 Pat. A. 72 J	12 400	70%	43	+ bis +++

Der Gehalt an saurer Phosphatase war in allen Fällen beachtlich hoch. Alkalische Phosphatase wurde in den leukämischen Zellen nicht gefunden. Die Indices der alkalischen Phosphatase der Neutrophilen sind in Tabelle I zusammengefaßt. Sie waren sehr unterschiedlich hoch zum Teil wurden die hohen Werte durch bestehende Infekte bedingt.

PAS-positive Substanzen waren in allen Fällen, wenn auch in unterschiedlichem Ausmass, feststellbar In 4 Fällen war die Anfärbung diffus und mässig stark. Ein Fall zeigte neben einer diffusen zytoplasmatischen Anfärbung in zahlreichen Zellen Granula, die teils fein, teils grob waren und sich manchmal in grossen Plaques darstellten

Auf Grund der Anfärbung bei den zytochemischen Reaktionen liessen sich die 5 Fälle in zwei Gruppen unterscheiden. Während Fall 1

ten der leukämischen Monozyten bei den verschiedenen zytochemischen Nachweisreaktionen
+ schwache ++ mäßig starke +++ sehr starke Reaktion

Naphthol-AS-Acetat Esterase	N-AS-E + 1,5 mg/ml NaF	Naphthylamidase	saure Phosphatase	alkalische Phosphatase	Naphthol-AS-D-Chloro-Acetat Esterase	Sudan-schwarz B	PAS	Peroxydase
++ bis +++	± bis +	+ bis +++	++ bis +++	0	0 bis +	0 bis +	± bis +	0 bis +
++ bis +++	± bis +	+ bis +++	++ bis +++	0	0 bis ++	0 bis +	± bis +	0 bis +
++ bis +++	/	/	++ bis +++	0	0 bis +	0 bis +	± bis +	0 bis +
++ bis +++	±	+ bis ++	+ bis +++	0	0	0 bis +	0 bis +	0 bis +
+ bis +++	± bis +	0 bis ++	++ bis +++	0	0 bis +	0 bis +	0 bis +	0 bis +
+ bis +++	± bis +	0 bis ++	++	0	0 bis +	0 bis +	0 bis +	0

2 und 3 eine hohe Aktivität der unspezifischen Esterase in den leukämischen Monozyten und Monozytenvorstufen zeigen, wiesen die beiden anderen Fälle einen deutlich niedrigeren Gehalt auf. Dieser geringere Fermentgehalt kann mit einer gewissen Berechtigung als Zeichen einer mangelnden Ausdifferenzierung betrachtet werden. Tatsächlich waren in den beiden letzten Fällen zahlreiche unreife Zellen im peripheren Blut feststellbar.

Um die Reaktionsintensität in den einzelnen Zellen einer Population besser zu erfassen, wurden jeweils 500 Zellen ausgezählt und je nach der gefundenen Reaktion in 4 Klassen eingeteilt (Tab. II). Besonders bei der Esterasereaktion zeigte sich deutlich, wie in den Fällen 1, 2 und 3 die stärker positiven Formen überwiegen, während in den Fällen 4 und 5 die Mehrzahl der Zellen schwächer reagiert.

Tabelle II Reaktionsstärke in den leukämischen Monozyten bei verschiedenen Bausteinen
0 keine + angedeutet bis schwache ++ mäßig starke

	α-Naphthyl-Acetat-Esterase				Naphthol-AS-Acetat-Esterase			
	0	+	++	+++	0	+	++	+++
Fall 1 Pat. R. 25 J	0	4	21	75	0	2	40	58
Fall 2 Pat. L. 60 J	0	5	27	68	0	8	30	62
Fall 3: Pat. B. 39 J	0	2	29	69	0	3	37	52
Fall 4 Pat. W. 44 J	0	12	38	50	0	42	46	12
Fall 5 Pat. A. 72 J	0	22	63	15	0	53	42	5

Diskussion

Normale Blutmonozyten sind auf Grund neuerer Untersuchungen in ihrer baustein- und enzymzytochemischen Ausstattung weitgehend charakterisiert. Ihre eindeutige Identifizierung in Ausstrichen von Blut und Knochenmark, sowie in Exsudaten und Infiltraten ist mit verfeinerten zytochemischen Methoden möglich [4, 12, 14, 15, 29, 36]. Die Theorie der Herkunft der Monozyten aus dem Knochenmark wurde mit diesen Methoden erneut untermauert, und die Abgrenzung dieser Zellen gegenüber dem retikulohistiozytären System ermöglicht [4, 14, 30, 31].

Es erscheint nun naheliegend, diese zytochemischen Eigenschaften auch als diagnostische Kriterien für die Erkennung der Monozytenleukämie heranzuziehen [11, 12, 14, 17, 20, 30]. In Tabelle I sind die wesentlichsten *kennzeichnenden Befunde zusammengefasst*, die bei der zytochemischen Untersuchung normaler Blutmonozyten feststellbar sind. Ein entsprechendes Verhalten der leukämischen Zellen muss verlangt werden, um die Diagnose einer Monozytenleukämie oder – falls es sich um jüngere *Zellen mit noch nicht so ausgeprägter* zytochemischer Ausstattung handelt, – einer Promonozytenleukämie zu stellen.

Unspezifische Esterase

Das Ausmass der mit den Substraten Naphthol AS-Acetat und α-Naphthyl-Acetat feststellbaren Esteraseaktivität in den verschiedenen

und enzymzytochemischen Nachweisverfahren. Jeweils 500 Zellen wurden klassifiziert. +++ sehr starke Reaktion, × nicht durchgeführt.

Naphthol-AS-Acetat Esterase + 1,5 mg/ml NaF				Naphthyl-amidase				Naphthol-AS-D-Chloracetat Esterase				Sudan-schwarz B			
0	+	++	+++	0	+	++	+++	0	+	++	+++	0	+	++	+++
20	80	0	0	10	16	22	52	12	70	18	0	96	4	0	0
×				×											
/	/	/	/	/	/	/	/	79	21	0	0	92	8	0	0
12	83	0	0	10	44	37	9	71	29	0	0	59	41	0	0
18	82	0	0	23	54	19	4	93	2	0	0	95	5	0	0
5	95	0	0	32	47	18	3	89	11	0	0	69	39	2	0

Zellformen stimmt nicht immer überein [14, 30]. Besonders ausgeprägt sind die Unterschiede bei verschiedenen hämatologischen Erkrankungen. So enthalten z. B. Plasmozytomzellen [18, 24], Erythroblasten bei erythrämischer Myelose [13, 30], bei Perniciosa [19] und bei toxischen Knochenmarkstörungen [30] und in Einzelfällen, was vielleicht zu wenig beachtet wird, auch Paramyeloblasten mehr α-Naphthylacetat Esterase als Naphthol AS-Acetat Esterase.





Abb. 2. Naphthylamidasereaktion. a) stark positive Monozyten. b) Promonozytenleukämie mit z.T. schwach positiven Zellen. 1000

Ein spezifisches Kriterium zur Erkennung der Monozyten wurde mit der Feststellung der fast vollständigen NaF Hemmbarkeit der NAS-E in diesen Zellen und ihren Vorstufen gegeben [6] In anderen Zellen des Blutes und Knochenmarks, mit Ausnahme ausgereifter Megakaryozyten, ist diese Esterase kaum hemmbar [30] Für die Erkennung monozytärer Leukämien legen wir demzufolge mehr Wert auf die hohe durch NaF hemmbare Aktivität der NAS-E als auf eine hohe α-NE, die auch bei anderen Erkrankungen feststellbar ist. Die von uns bisher gesehenen Monozyten und Promonozytenleukämien ließen neben einer hohen α-NE tatsächlich auch eine hohe, durch NaF fast vollständig hemmbare, NAS-E erkennen. Im Gegensatz dazu wurde die mäßig starke NAS-E leukämischer Promyelozyten und Plasmozytomzellen durch NaF kaum gehemmt, ebenso die geringe NAS-E der Myeloblasten- und Paramyeloblastenleukämie (Tab. I)

Naphthylamidase

Unter den Zellen des peripheren Blutes zeigen die Monozyten bei Verwendung des Substrates L-Leucyl-β-naphthylamid die stärkste zytochemisch nachweisbare Aktivität [32] Diese bleibt den Monozyten auch in Exsudaten, in Hautfensterpräparaten [29] und in Ergüssen [eigene Beobachtung] erhalten. Retikulumzellen enthalten viel Promyelozyten mäßig viel und die übrigen Blut und Knochenmarkszellen enthalten wenig Naphthylamidase [1 27 32 eigene Beobachtungen]

Bei Monozytenleukämien ließ sich das Ferment unter Verwendung von Leucin-β-naphthylamid in unterschiedlicher Intensität in den leukämischen Zellen nachweisen im Durchschnitt war die Aktivität mäßig hoch [1] In einigen Fällen fielen manche Zellformen durch ihren hohen Fermentgehalt auf. Sehr junge Zellformen – Monoblasten – enthielten ebenso wie Myeloblasten keine Fermentaktivität.

Naphthol AS-D-Chloroacetat Esterase

Dieses Ferment ist, von den stark positiv reagierenden Gewebsmastzellen abgesehen, für die Zellen der neutrophilen Reihe spezifisch Basophile und Eosinophile enthalten in der Regel keine entsprechende Fermentaktivität [16, 30] Die in den Monozyten manchmal nach-



Abb. 3. Naphthol-AS-D-Chloroacetat Esterase) nicht fermenthaltige leukämische Monozyten neben positivem neutrophilen Segmentkernigen; b) und) z.T deutlich positive leukämische Monozyten ( ) neben ngedeutet und schwach positiven (b) sowie stark positiver Promyelozyt (b) und Neutrophile ( ) b) und) selber Fall wie) und) der Abb. 1 1000 ×

weisbare geringe Aktivität wurde ebenso wie die Peroxydasereaktion der Monozyten, als Beweis für die enge genetische Beziehung der Monozyten zu den Zellen der neutrophilen Reihe angesehen [14]

Drei der beobachteten Monozytenleukämien (Nr 3 4 5) liessen keine Aktivität des Ferments in den Zellen erkennen. Der Fall 2 enthielt nur in vereinzelten Zellen eine angedeutete Aktivität. Hingegen waren die leukämischen Zellen des Falles 1 z.T stark positiv unterschieden sich allerdings deutlich von der sehr ausgeprägten Reaktionsintensität der wenigen vorhandenen reifen Promyelozyten, von denen sie sich im übrigen auch morphologisch völlig unterschieden Eine positive Korrelation zwischen dem Vorkommen reifer Promyelozyten im Blut und Knochenmark und stark N AS-D-E positiven Monozyten konnten wir nicht feststellen

Ähnlich den normalen Promyelozyten weisen auch die Zellen der Promyelozytenleukämien einen hohen Behalt an N AS-D-Cl E auf

Myeloblasten und Paramyeloblastenleukämien enthalten, wenn überhaupt, eine sehr niedrige Aktivität des Fermentes.

Sudanschwarz B-Färbung

Übereinstimmend zeigten in allen Fällen die leukämischen Zellen einen geringen oder keinen Gehalt an Sudanschwarz-anfärbbaren Substanzen. Bei den wenigen festgestellten stärker positiven Zellen dürfte es sich um Promyelozyten gehandelt haben. Die leukämischen Monozyten stimmten in ihrem Verhalten mit normalen Blutmonozyten überein, die in der Regel eine geringe Anfärbbarkeit mit Sudanschwarz B erkennen lassen. Im Gegensatz dazu weisen Promyelozytenleukämien eine deutlich grössere Anzahl positiver Zellen und auch einen stärkeren Reaktionsgrad der Einzelzellen auf.

Für die Auswertung zytochemischer Reaktionen an hämatologischen Präparaten wurde in Anlehnung an die score-Technik zur Be-



Abb. 4 Sudanschwarz B-Färbung) z. T. angedeutet positiv und b) völlig negativ leukämische Monozyten. Eine Anfärbung zeigen in) ein eosinophiler Granulozyt und in b ein neutrophiler Myelozyt. 1000 ×

urteilung der alkalischen Neutrophilenphosphatase mehrfach eine ähnliche Technik verwendet [20 34]. Es scheint uns für diese Fälle allerdings zweckmässiger, die Reaktionsintensität einer bestimmten Zellpopulation aufgeschlüsselt nach Reaktionsklassen in Prozenten anzugeben, wie wir es in Tab. II durchgeführt haben. Diese Darstellung gestattet eine bessere Beurteilung der gefundenen Aktivität und des Grades der Ausdifferenzierung der leukämischen Zellen unter der doch wahrscheinlichen Annahme, dass der Fermentgehalt der zunehmenden Differenzierung parallel geht. Bei reaktiven Monozyten im Rahmen von Stammzellenleukämien sind die Monozyten durch eine hohe, Stammzellen durch eine fehlende oder sehr geringe Esteraseaktivität gekennzeichnet. Es fehlen Zwischenstufen, die hingegen bei Monozytenleukämien vorhanden sind [14]. LEDER [14] entwickelte neuerdings die Theorie, die bisher als «jüngere» und deshalb fermentärmere Zellformen angesehenen leukämischen Zelltypen, z.B. «Myeloblasten» im Endstadium von Monozytenleukämien, seien das Resultat einer Entdifferenzierung der ursprünglich gewucherten Monozyten, etwa analog dem Vorgang der Entdifferenzierung bei Karzinomzellen. Ein Beweis für diese Ansicht steht noch aus.

Zusammenfassung

Auf Grund zytochemischer Untersuchungen lässt sich das Krankheitsbild der Monozytenleukämie eindeutig abgrenzen. Die gewucherten Zellen leiten sich von im Knochenmark gebildeten Blutmonozyten ab. Die Diagnose der Monozytenleukämie erfolgt auf Grund folgender zytochemischer Kriterien: 1. Der hohe Gehalt der leukämischen Zellen an unspezifischer Esterase bei der Verwendung von Naphthol-Acetaten als Substrat. Substrate der Naphthol-AS-Reihe sind besser geeignet als α-Naphthyl-Acetat, das ersteres auch in Paramyeloblasten eine positive Reaktion gibt. 2. Die Hemmbarkeit der starken Esteraseaktivität durch NaF (1,5 mg/ml) unter Verwendung der Substrate der Naphthol-AS-Reihe; 3. Eine mässige bis starke Aktivität der Naphthylamidase in den leukämischen Zellen. 4. Die geringe Anfärbbarkeit mit Sudanschwarz B. 5. Eine in der Regel geringe Aktivität der Naphthol-AS-D-Chloroacetat-Esterase in den leukämischen Monozyten.

Summary

Monocytic leukaemia can be clearly distinguished on the basis of cytochemical studies. The hyperplastic cells are derived from monocytes formed in the bone marrow. The following cytochemical criteria determine the diagnosis of monocytic leukaemia: (1) The high level of unspecific esterase in the leukaemic cells when naphthol acetates are used as substrate. Substrates of the naphthol-AS series are more suitable than alpha-naphthyl acetate, which occasionally gives positive reaction also in paramyeloblasts. (2) Inhibition

of the marked esterase activity by NaF (1.5 mg/ml) when naphthol-AS substrates are used (3) Moderate to marked naphthyl amidase activity in the leukaemic cells; (4) Poor staining with Sudan black B (5) Usually limited naphthol-AS chloroacetat esterase activity in leukaemic monocytes.

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which with acute terminal blastic crisis, chronic lymphoid leukemia (6), reticular (9) and lymphoid (3) disseminated sarcomatous diseases, Hodgkin's disease (12) and plasmocytoma (6). All these patients have been studied out of phases of hematologic or clinical remissions, generally in the first days after the admission in our Clinic. Moreover 6 patients with epithelial neoplasias and 9 with *systemic lupus* erythematous have been studied.

Since the anti-I serum used in our researches has been obtained from O-group patient, all the subjects included in this study were selected belonging to this erythrocyte group.

2) In vitro-interaction between Mycoplasmas and erythrocyte I antigen. The red cells of two normal O-group I-positive subjects have been suspended, after three preliminary washings, in sterile saline at 2% concentration, and incubated, respectively for 60 min to 22°C and for 24 h to 4°C with the following human Mycoplasma strains: Mycoplasma *pneumoniae* (Eaton agent) (FH strains), Mycoplasma fermentans (PG 18), Mycoplasma hominis type 1 (PG 21) and type 2 (PG 27), Mycoplasma orale (Patt strain). They have been grown (3–5 days at 37°C, for M. pneumoniae 10 days) in PPLO fluid medium [ALTUCCI *et al.*, 1966], then centrifuged 1 *h* at 12 000 rpm, and finally resuspended in saline at 100-fold concentration.

At the end of these two incubation periods, the red cell suspensions have been washed three times with sterile saline, resuspended in the same medium at 2% concentration, and were tested with the anti-I serum, adding 0.1 ml of the red cell suspension to 0.1 ml of the serum. The degree of the I positivity of erythrocyte suspensions after the incubation with Mycoplasmas has been compared with that of the same suspensions before the addition of these micro-organisms.

3) Interaction between Mycoplasmas and I antigen in vivo. We have selected for this study the hamster infection with M. pneumoniae [7]. At first, we must remember that starting researches, performed for testing the behaviour of I antigen in the normal hamster red cells, have always shown (100% of the experiments) marked I-positivity in the erythrocytes of these animals.

We *have infected, in two different experiments,* 4 hamsters, intranasal route (ml 0.1 × 2) previous Nembutal narcosis, with *M. pneumoniae* FH strain (10^6 CFU/ml). Two animals were sacrificed in the 10th day for reisolating on agar the infecting agent; and 2 respectively in 20th day for measuring the specific complement fixing antibodies.

For all the sacrified animals we have performed lung pathology evaluation, agar titration, at tenfold dilutions, of infecting agent, I-antigen research in the red cells, serum cold agglutinins [FELLER and HILLEMAN] and CF antibodies measurement [5, 1], reticulocyte count and Ht determination.

Results

All the red cells of patients suffering from leukemia or other malignancies have presented a strong *I* positivity. We could not show any difference, with both types of incubation, between these erythrocytes and those from controls. As registered in table I, we have always

Coming the serum we have used from O-group patient, we have examined, both for (1) and (2), all O-group red cells. It has been said [15] that Mycoplasmas are more active toward I antigen from A-group subjects; nevertheless, the same AA. admitted the possibility of an interaction (of biophysical and/or enzymatic order) [4] between Mycoplasmas and O-group cells.

The specificity of this phenomenon was demonstrated through the lack of agglutination of hamster red cells by both anti-A and anti-B serum.

Table I. Intensity of I-positivity of red blood cells of normal subjects, patients suffering from leukemia, malignancies and systemic *lupus erythematosus*

Groups	No. of cases	Anti-I serum agglutination	Intensity
Normal subjects	10	positive	+++
Acute leukemia	15	positive	+++
Chronic myelogenous leukemia	15	positive	+++
Chronic lymphoid leukemia	6	positive	+++
Reticulosarcoma	9	positive	+++
Lymphosarcoma	3	positive	+++
Hodgkin disease	12	positive	+++
Plasmocytoma	6	positive	+++
Epithelial neoplasias	5	positive	+++
Systemic *lupus erythematosus*	9	positive	+++

observed an I positivity with 3 + intensity comparable to that of controls. The same results were obtained in epithelial neoplasias and systemic *lupus erythematosus*

The red blood cells of normal subjects, after the two different incubation periods with the Mycoplasmas, have shown neither a fall nor a loss of I positivity The intensity of the agglutination by anti-I serum has been equal both for the red cells incubated and for those not incubated with Mycoplasmas (table II)

Although the infection by *M pneumoniae* in hamster has been documented by the pathological findings in the lungs, by the isolation (in 10th day) of Mycoplasma, and by the content in CF antibodies (in 20th day) of the sera of the animals, we have not been able to show any change of the normally high I-positivity in the hamster red cells. The research of cold agglutinins has been negative the reticulocyte count and the Ht value have not shown any difference in respect to the controls.

Discussions and Conclusions

Our results about the eventual loss of I antigen in red blood cells of patients affected by leukemia or other systemic and malignant conditions, and about the capacity of Mycoplasmas to inactivate I antigen *in vitro* or *in vivo* have been negative. They do not agree with the conclusions of McGinniss *et al.* [12], Schmidt *et al.* [14 15] Barile *et al.* [4] On the other hand, during the development of our work,

Table II. I-positivity of normal red blood cells before and after incubation with Mycoplasmas *in vitro*

Normal red blood cells samples examined	Mycoplasma strains tested	Red blood cells incubated at 22°C for 60 min and at 4°C for 24 h Intensity of I-positivity before incub.	after incub.
1	*M. pneumoniae* (FH)	+++	+++
	M. fermentans (PG 18)	+++	+++
	M. hominis 1 (PG 21)	+++	+++
	M. hominis 2 (PG 27)	+++	+++
	M. orale (Patt)	+++	+++
2	*M. pneumoniae* (FH)	+++	+++
	M. fermentans (PG 18)	+++	+++
	M. hominis 1 (PG 21)	+++	+++
	M. hominis 2 (PG 27)	+++	+++
	M. orale (Patt)	+++	+++

our data have been confirmed by the results of other authors [8, 13 10]

It is not easy actually to find an explanation for these opposite results. From the technical point of view the most accurate works appear those of Dubos *et al* [8] Salmon *et al* [13] and above all, by Feizi and Hardisty [10] who have performed a quantitative study of I antigen in red cells, and [at least Feizi and Hardisty] have tested both leukemic and control red cells against three different anti-I sera (whose two with high titer)

An interesting observation [10] is related to the different capacity of the various anti-I sera to detect a partial loss of I antigen (as, for example, that of some types of newborn red cells, I-cord) However it is not probable that this explanation may also partially justify the different results. On one hand it is clear that all the anti-I sera are able to show the complete loss of I antigen and/or its lack in the rare 'i' cells in adult subjects [10] On the other hand, the loss of I antigen in leukemic red cells [12 14] has been observed with high concentrations of anti I serum (1 2)

In our work, we could not perform a quantitative titration of anti-I reactivity of the various red cells examined, because of the low titer of the serum. Nevertheless, the intensity in the agglutination with 1 2 serum dilution clearly showed the normal presence of I antigen in the red cells. On the other hand, the technique followed by us is the same of the authors, who, on the contrary have obtained positive results. The

Table III Behaviour of I-positivity related with other tests, in hamsters infected by *Mycoplasma pneumoniae*

Testing criteria	Normal hamsters sacrified	Hamsters infected with *M. pneumoniae* Sacrif. 10th day	Sacrif. 20th day
Lung pathology	absent	1) interstitial pneumonia 2) interstitial pneumonia	1) healing lesions 2) healing lesions
Agar titration of *M. pneumoniae* (CFU/ml)	n. d.	1) 2.10^{7} $1.8.10^{7}$ 2) $1\ 10^{7}$; $0.5.10^{7}$	1) $1.5.10^{4}$; $1\ 10^{4}$ 2) 0 2.10^{4}
I-positivity	+++	1) +++; +++ 2) +++; +++	1) +++;+++ 2) +++ +++
Cold agglutinins	n. d.	1) absent 2) absent	1) absent 2) absent
Specific CF antibodies	n. d.	1) <10 <10 2) 10; <10	1) 40 80 2) 40; 40
Reticulocytes, ‰	17 22‰	1) 20 20 2) 19 23	1) 19 18 2) 17; 18
Hematocrit, %	40–42	1) 38; 43 2) 40 43	1) 40; 43 2) 41 42

Expressed as the reciprocal of the highest serum dilution still able to show complete absence of hemolysis with 4–8 units of antigen and 2 full units of complement.

discrepancies with the results of these authors, obtained with *in vitro* incubation between Mycoplasmas and I positive red cells are – nevertheless – partial. They concern *M. pneumoniae* (active, following American authors, in determining I negativity of erythrocytes) and *M. fermentans* (only slightly active). Of other 3 strains tested by us, *M. hominis* 1 has been inactive also in their experiments, *M. hominis* 2 variably inactive or slightly active, *M. orale* not tested. Contrarily to SCHMIDT *et al.* [14] we have not previously disrupted Mycoplasmas in these experiments by freezing and thawing twice. It is not easy to say how this difference may have contributed to give a different result.

More recently during the writing of our work, FEIZI and DARRELL [9] have reported that the treatment of OI red cells with two different strains of *M. pneumoniae* did not affect the activity of any of three anti I sera tested either after overnight incubation at 4 C, or after incubation at 22° C. These data agree with our results.

Also the experiments concerning the behaviour of I antigen *in vivo* after a documented Mycoplasma infection have given conflicting results. On one hand, American authors [16 12] have recently noted

that the activity of the erythrocyte I agglutinogen was (only) transiently reduced following *M. pneumoniae* infection in 5 of 27 volunteers, without signs of hemolytic anemia. On the other hand, Feizy and Darrell [9] have found that the red cells from patients with *M. pneumoniae* infection (1st 3rd 7th week) reacted with the anti-I serum in a manner indistinguishable from the normal cells.

We have obtained similarly negative results in *M. pneumoniae* infection in the hamster.

However it must be stressed that we cannot say at present, whether so conflicting results, in the whole, may depend also from the eventually different antigenic distribution of various I positive red cells, and/or from the different source of anti I antibody in human sera tested (a natural agglutinin in I negative patient or a cold agglutinin from patients who had suffered or not for a *M. pneumoniae* infection). Also Smith *et al.* [16] admit that the greater sensitivity of the cold agglutinin anti I reagent than the iso-anti I reagent in detecting these antigenic changes may have been due to basic differences in molecular structure of anti I cold agglutinins found in cold agglutinin disease and iso anti-I cold agglutinins.

In conclusion, our data bring us to exclude both the changes in I-positivity of leukemic red cells and the interaction between I-antigen and Mycoplasmas. It cannot be excluded that Mycoplasmas are the principal microbial candidates for the triggering of autoimmune conditions [8]. Nevertheless, our actual results bring us to consider with relative scepticism, also from this point of view the possibility of an etiological relationship between Mycoplasmas and leukemia.

Summary

The red cells of patients affected by leukemia and other systemic or malignant diseases do not show the loss of I antigen after two different types of *in vitro* incubation with anti-I serum. Normal red cells, incubated *in vitro* with five different human Mycoplasma strains, retain their normal I-positivity.

M. pneumoniae infection in hamsters does not change the I positivity of the red cells. The results of some authors, who showed the loss of I antigen in leukemic red cells and its interaction *in vitro* with Mycoplasmas, are discussed in relation with our negative results.

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separately were prepared for histological and chromosomal study and the remaining tissue was then transplanted into syngeneic hosts.

3. Thymus from mice exposed to thigh-shielded irradiation. Eight weeks following the first in series of thigh-shielded irradiation, mice were killed and pieces of the thymus implant prepared for histological, chromosomal and transplantation study as mentioned above.

4. Virus-induced lymphomas.) Four 7 and 10 weeks after inoculation of virus into the intrarenal implant in the thymectomized and irradiated recipients the animals were killed and pieces of the thymic implant prepared for histological, chromosomal and transplantation study as mentioned above.

b) In a separate set of experiments, chromosomal analysis and histological examination were performed on 11 virus-induced disseminated lymphomas. No transplantation was performed on these animals since other experiments have shown that the *disseminated* lymphomas are readily transplantable (unpublished data).

Results

1. Thymus from normal controls. The chromosome pattern of thymus cells from 7 normal control mice of various ages is shown in table I. A metaphase of a normal thymus cell is shown in figure 1. Occasional cells contain only 39 chromosomes and a few cells have an extra chromosome. Although an attempt was made to count only cells that appeared intact, the possibility cannot be excluded that the missing and extra chromosomes are artefactual, due to excessive hypotonic treatment.

2. Thymus from mice exposed to leukemogenic whole-body irradiation (table II). Eight weeks after irradiation the thymic lobes were very small. Two were *histologically classified* as *atrophic, indicating* lymphocytic depletion [7]. The small size of these lobes made it dif

Table I. The chromosome pattern of normal untreated mice

Sex	Age weeks	No. cells with indicated chromosome number 39	40	41
F	6	1	38	
F	10		42	
F	21	1	36	1
M	20	2	36	1
M	20		41	
M	20	1	29	1
M	21	1	33	

Table II. The chromosome pattern and transplantation behaviour of thymus cells during the development of radiation-induced lymphomas

Weeks after X-ray	Mouse No.	Histol. classific.	Results of transpl.	No. cells with indicated chromosome number <38	38	39	40	41	42	>42	80	Structural chromosome aberrations	Final interpretation normal	equivocal	definitely abnormal
8	1	Atrophy				2	8	1					×		
	2	LCT				1	8	1					×		
	3	Normal			1	1	12					+		×	
	4	Normal				2	11						×		
	5	LCT			2	2	9	1				+		×	
	6	Atrophy		2	1		8		2	2		+		×	
14	1	LCT		1		1	24	2	1					×	
	2	LCT				2	17	2	1	4					×
	3	LCT				3	24	1	3						×
	4	IL	+	1	1	4	17		2	3		++			×
	5	IL		2	2	2	15	1						×	
	6	IL			2	7	18	4				+			×
20	1	LCT				1	11	2		5				×	
	2	IL			1	2	17	1						×	
	3	IL	+		4	7	4			2				×	
	4	IL	+	1	2	1	10			1				×	
	5	IL	+		1	4	13		1					×	
	6	IL			3		9		1	1		+		×	
	7	IL	+	1	2	2	28	2		1					×
	8	IL		1	2	2	12		1	4				×	
	9	IL	+	1	2	1	16	2	1	7	6				×
	10	IL		1	2	1	15	2						×	

LCT lymphoma confined to thymus, *IL* invasive lymphoma.

ficult to obtain good chromosome preparations and only relatively few metaphases could be examined. Occasional morphological aberrations were observed and in one atrophic lobe (No. 6) 7 of 15 cells showed aneuploidy. All of the implanted thymic lobes gave a negative transplantation test at 8 weeks after irradiation.

Fourteen weeks after irradiation all the lobes exhibited histological signs of tumour either as lymphomas confined to the thymic lobe or as invasive lymphoma. Only one, however, gave a positive take after transplantation. All these lobes showed numerical aberrations in chromosome pattern (fig 2) and some chromatid breaks were observed. Four of the 6 animals were classified as 'definitely abnormal'.

At 20 weeks all lobes examined were histologically lymphomatous. Though the number of metaphases examined is small in some of the lymphomas, it is obvious that all tumours analyzed at 20 weeks had substantial proportions of aneuploid cells. Two out of 10 lobes were classified as 'definitely abnormal'. Six of 36 cells from one mouse (No. 9) had chromosome numbers in the tetraploid range. Only occasional breaks were observed in these groups.

Five out of 10 lobes gave a positive transplantation test (table II). All of these were invasive lymphomas in a macroscopically and microscopically early stage. Mice No. 2 exhibited slight dissemination to distant organs. Thus no correlation was found between specific morphological features and positive transplantation tests of the thymomas developing after irradiation. Nor was there a correlation between transplantation behaviour and the first appearance of numerical or morphological abnormalities in the chromosome patterns.

3. Thymus from mice exposed to thigh-shielded irradiation. Following irradiation with the thighs shielded the thymus undergoes characteristic histological alterations: instead of the atrophy preceding the tumour development following leukemogenic whole-body irradiation, the thigh-shielded irradiation is attended by rapid recovery and hyperplasia of the thymus that becomes populated by small dense thymocytes [7]. Good spreading of the chromosomes is very difficult to obtain from these cells and a total of 40 acceptable metaphases could be obtained from 4 mice. The number of the chromosomes of these cells are presented in table III showing no major deviations from the mode of 40. No structural abnormalities were observed and the transplantation tests were all negative.

4. Virus-induced lymphomas (table IV). Four weeks after the inoculation of leukemogenic virus all of the three implants available for

Table III The chromosome pattern of thymus cells following thigh-shielded irradiation of the mice

No. cells with indicated chromosome number				Final interpretation		
38	39	40	41	normal	equivocal	definitely abnormal
2	4	35	1		×	

The table includes the chromosome counts of thymus cells from 4 mice

histologic examination were neoplastic. Two of these gave positive transplantation tests. Occasional aneuploid cells occurred but no convincing chromosomal aberrations were encountered. One of 4 were considered equivocal

Seven weeks after virus-inoculation all implants were neoplastic and exhibited histological evidence of invasive lymphoma although in an early stage of development. One of these, No 1 had about 30 of the cells in the hyperdiploid range Because of the very small size of this tumour few metaphases could be examined The other animals in these groups showed occasional loss of chromosomes but no major numerical or morphological abberrations occurred. Because of the small number of metaphases accessible to analysis the majority of these cases were classified as equivocal' All tumours gave positive takes after transplantation.

Ten weeks after virus-inoculation the tumours were histologically still in an early stage of development. Two of the lymphomas were still non-in vasive. The chromosome counts indicate numerical aber rations in most of the cases, except for No 3 and possibly No. 2. One tumour No 5 had some missing chromosomes and one tetraploid cell and occasional chromatid breaks were observed. Four of the 5 tumours were classified as equivocal' Three implants, of which 2 were histologically classified as lymphoma confined to the implant, yielded positive transplantation takes. One invasive lymphoma with 5 out of 16 cells in the hyperdiploid range was negative after transplantation.

The chromosome pattern of virus-induced, disseminated lymphomas in the terminal stage of the disease is demonstrated in table V The counts indicate numerical abnormalities in all cases and 9 of the 11

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Double Blind Evaluation of the Haemostatic Effect of Adrenochrome Monosemicarbazone, Conjugated Oestrogens and Epsilonaminocaproic Acid after Adenotonsillectomy

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Evaluation of the haemostatic effect of agents designed to reduce blood-loss is beset with numerous difficulties. Patients with a chronic haemorrhagic diathesis often bleed intermittently, haemorrhage ceasing or diminishing regardless of the drug administered. If the alleged beneficial effect of a haemostatic agent is tested when normal haemostasis is challenged, it must be ascertained that the severity of bleeding and underlying mechanism leading to it are similar in the different individuals. This latter requirement implies that traumatized persons or patients subjected to the multiple variants of surgery cannot be considered as valid test material for this purpose.

A more uniform challenge to haemostasis is a well standardized bleeding time, but even in the best hands it is difficult to make a uniform cut through the skin, due to the natural anatomical variations in vessel size. Because of other variables also, numerous cuts (e.g. 20 in the rabbit's ear) have to be made to ensure statistical validity [11]. Reproducible results are also very difficult to obtain in man [13].

In view of these drawbacks we chose adenotonsillectomy as a test situation wherein the mechanical trauma is fairly standard and large enough to permit *pharmacologically induced reduction in blood loss*, if any, to be measured. Furthermore a large number of individuals can be included in the trial and as only healthy children are involved, the subjects can be considered as constituting a fairly homogenous group. The type of operation selected also offers the possibility of assessing blood-loss.

Three substances commercially available as haemostatic agents were evaluated in double blind trials using measured blood-loss as criterion in children subjected to adenotonsillectomy The order of allocation of the test substance or placebo was randomly determined.

Methods

The experiments reported herein were performed in collaboration with the ENT department of the University Hospital of Louvain, Belgium The subjects involved were all patients admitted to this department the night before operation and discharged 10 h after surgery The technique used for tonsillectomy and adenoidectomy did not vary substantially among the different surgeons of the University department where all the operations were performed. Minor variations could not influence the results as control group was included in the series. No premedication was given the child remained seated during the operation and was briefly anaesthetized with ethyl chloride. The adenoids were removed using the Vollmann curette and tonsillectomy performed by means of the Sluder tongs 80% of the children studied were subjected to both operations.

In the trial with adrenochrome monosemicarbazone, loss of blood through aspiration or ejection, was ascertained gravimetrically Previously weighed dry gauze sponges, doctors and nurses' blouses and bedlinen were used and the quantity of blood-loss determined by immediate re-weighing. The blood lost during the following 10 h was assessed using the same gravimetric method. The incidence of saliva and other ejected material in the weight difference can be disregarded because large control group underwent similar treatment.

In the trials with conjugated oestrogens and with epsilon-amino-caproic acid, total blood loss was determined by placing all the ejected blood, tampons, towels, doctors and nurses' blouses and bedlinen used for one child in blood-loss monitor The instrument used was the Perdometer (A.B. Lars Ljungberg, Stockholm). The blood containing materials were collected in tank containing 40 l of water and haemolysing agent. The blood was washed out by rotary washing mechanism and haemolysed. The blood-containing solution was filtered and pumped continuously through photometer that measured the light absorption due to haemoglobin content. Since this is proportional to the amount of blood present, the quantity of blood lost can be immediately ascertained by conversion table that takes into account the patient' preoperative haemoglobin value. The objection that swallowed blood is not taken into account can be disregarded because the same procedure was used for both the treated and control groups. The measurement of very small amounts in the Perdometer is not reliable below 10 ml of blood due to uneven distribution of fluids in the instrument (40 l) but abov this figure, the correlation with the measured value is good. The average error of the instrument in test determinations of known amounts of blood was 3% [14]. Haemoglobin was determined by the method of DRABKIN.

Adrenochrome monosemicarbazone (1-methyl-2 3-dihydro 3-hydroxyindole-5, 6-quinone) (Adrenoxyl® Labaz Adrenosem® SE Massengill Co.) is one of the first derivatives produced during the oxidation of epinephrine, the semicarbazone of adrenochrome is stable substance. Vials containing 5 mg active substance or placebo were prepared. The water soluble conjugated oestrogens (Premarin® Ayerst; Equigyne® Toraude) were used. Each vial contained 20 mg natural oestrogen in the dried state or placebo. The epsilonamino-caproic acid-vials (EACA) were Epsilapron® Kabi, Stockholm, containing 5 ml active substance at 40% (w/v)

The kind cooperation of the doctors and nurses of the ENT Department (Head Prof. Dr FR. CRAMER) is gratefully acknowledged.

Results

Adrenochrome

Vials with adrenochrome monosemicarbazone (5 mg) or placebo, identical in appearance, were prepared and numbered at random by the manufacturer and administered according to the double blind technique. The key indicating the content of each vial was kept in a sealed envelope which was not opened until the trial had been completed. A total number of 100 children took part in the trial of which 6 were eliminated at the end of the study because of incomplete data. Placebo was administered to 47 children and adrenochrome monosemicarbazone to the remaining 47 cases. Each patient received the night before amygdalectomy 5 mg adrenochrome intramuscularly and a second identical dose approximately half an hour before operation. This dose is considered to be high for children with an average weight of 22.3 kg.

In this trial, blood-loss was measured by a gravimetric method. The mean difference in weight of the linen before and after operation was 60 g (SD 21) in the adrenochrome-treated group and 58 g (SD 28) in the placebo-treated group. Both groups were similar in weight and height (table I).

Conjugated Oestrogens

This trial also was conducted strictly according to the double blind method, the sequence of substances given to the patients being determined by random allocation. All vials were similar in appearance. The content of one vial (20 mg) was injected intramuscularly approximately half an hour before the operation. At all 200 children were involved in this trial; upon completion of the study 27 cases (15 treated with conjugated oestrogens and 12 with placebo) had to be rejected for a variety of reasons likely to invalidate the results (loss of part of the linen, spitting of blood in the toilet or children leaving the ENT department before 6 p.m.). After disclosure of the code-key it appeared that, of the remaining 173 children, 85 received the test substance and 88 the placebo material.

The results of measured blood-loss are given in table I. Both groups of patients were identical in weight and height, no difference in blood loss could be found.

Table I

	Monosemicarbazone of adrenochrome	Placebo
Number of children in the trial	47	47
Mean blood loss	58 g (SD 28)	60 g (SD 21)
Mean height	115 cm (SD 0.12)	112 cm (SD 0.18)
Mean weight	22.2 kg (SD 7.7)	22.2 kg (SD 9 7)
	Conjugated oestrogen	Placebo
Number of children in the trial	85	88
Mean blood-loss	65.4 ml (SD 37.8 ml)	89.8 (SD 37.0)
Mean height	117 cm (SD 16.1 cm)	115 (SD 13.7)
Mean weight	22.6 kg (SD 8.4)	21.2 kg (SD 8.06)
	EACA (2 g I.)	
Number of children in the trial	25	
Mean blood-loss	89.0 ml (SD 36.6)	
Mean height	121.5 cm (SD 15.06)	
Mean weight	23.9 kg (SD 6.8)	
	EACA	Placebo
	≤ 15 kg: 4 g I. > 15 < 30 kg 8 g I.v > 30 kg: 12 g I.	
Number of children in the trial	31	36
Mean blood-loss	60.3 ml (SD 17)	87.2 ml (SD 40.0)
Mean height	119 cm (SD 16.2)	125 cm (SD 13.4)
Mean weight	22.3 kg (SD 7.2)	22.6 kg (SD 8.3)

Epsilon-Amino-Caproic Acid

Vials containing 5 ml EACA at 40% were available. The content of one vial (2 g) was injected intravenously 30 to 60 min before operation in 25 children. There was no control group however the children involved in this trial were not preselected. The results of blood-loss are compared with the blood-loss obtained in the 88 control children, operated in identical circumstances. No difference was noted in measured blood-loss 89 0 ml (SD 36 6) in the treated group 89.8 ml (SD 37.0) in the control group of the previous trial.

As the possibility that the EACA dose was insufficient could not be excluded, it was decided to initiate a new trial whereby the EACA dose was increased and adapted to the weight of the child. The following doses of EACA were administered intravenously 30 to 60 min before the operation in children less than 15 kg 4 g EACA between

15 and 30 kg 8 g EACA over 30 kg 12 g EACA. A few instances of vomiting were observed after injection of the drug

As the EACA dose had to be adapted according to the weight of the child, this part of our investigation was not a double blind but still a controlled not stratified study Each patient involved in the study was allocated to the EACA or placebo group according to serial numbering before the trial started each number was listed at random in one of the two groups. As a significant difference in blood loss was observed after treatment of 67 children (31 in the EACA group, 36 in the control group) this trial was not continued with the remaining 33 of the 100 children initially envisaged Both groups were similar with respect to weight and height. The blood loss in the EACA treated group was 60.3 ml (SD 17) and in the control group 87.2 ml (SD 40 0 ml) this difference is highly significant ($P < 0\ 001$)

Discussion

The alleged haemostatic effect of conjugated oestrogens is based on the work of Schiff and Burn [17] These authors have demonstrated a progressive change from the sol to the gel state of mucopolysaccharides in the ground substance around small vessels and in the arterial walls. This polymerization of the mucopolysaccharides was observed in hamsters, monkeys and men following intravenous administration of conjugated oestrogens.

Detailed reports concerning the impact of conjugated oestrogens on blood coagulation are rare and often conflicting An elevation of fibrinogen [12] prothrombin and factor V and a decrease of antithrombin [6] have been described but these findings could not be confirmed by other investigators [7 9] It is possible however that capillary oozing could be shortened by action of oestrogens at the vascular or extravascular level without being influenced by the intravascular coagulation factors. An attentive study of the clinical reports is rather disappointing as the effect of conjugated oestrogens on haemostasis is based on clinical judgment only except in two trials conducted according to the double blind method which however were both negative [7 19]

It is beyond doubt that epinephrine from which monosemicarbazone of adrenochrome is derived has a vasoconstrictor effect on the microcirculation The direct relationship between the decreased flow

of blood within these vessels and its alleged function in lessening blood loss is less clear. As epinephrine is readily oxidized to adrenochrome which itself is also unstable, a more suitable agent for clinical use was developed: the monosemicarbazone of adrenochrome which is said to be stable when administered by mouth or by injection [4].

Numerous studies have been published on monosemicarbazone of adrenochrome claiming its increased capillary resistance and usefulness in reducing haemorrhage after oral or parenteral administration. Unfortunately the enthusiastic clinical reports on its haemostatic role in man originate from clinical impressions and/or uncontrolled clinical trials. The only reported double blind clinical trial failed to confirm previous favourable reports: in this trial the observation of the duration of bleeding after dental extraction was made by the patient himself, thus exposing the evaluation method to criticism because of the ill-defined subjective criterion [5]. In another controlled trial the same drug had no statistically significant effect on capillary resistance as measured by a negative pressure method. No apparent difference in the incidence of post-operative hyphaema in a series of forty pairs of patients, one of each pair receiving monosemicarbazone of adrenochrome by mouth, was observed [18].

Different fibrinolysis inhibitors are commercially available, some of them being extracted from tissue. The polypeptide isolated from bovine lung (Trasylol® Bayer) or pancreas (Iniprol® Choay) are inhibitors of trypsin, plasmin and plasminogen activator. Synthetic fibrinolysis inhibitors are the aminoacids epsilon amino-caproic acid (EACA) Transamino-methyl-cyclohexane-carboxylic acid (AMCHA or tranexamic acid: cyclocapron) and para-aminomethyl benzoic acid (PAMBA).

It has been demonstrated beyond reasonable doubt in controlled studies that synthetic fibrinolysis inhibitors can diminish bleeding from areas with increased local fibrinolytic activity as for instance in the urinary tract [1, 8, 16] and uterus [10, 15, 20]. The effective oral dose required is approximately 400 mg/kg EACA or between 60–90 mg/kg AMCHA.

Activators of the fibrinolytic system are present in almost all tissues as shown by the experiments of ASTRUP and PERMIN [3]. After adenotonsillectomy haemorrhage at the adenoid and tonsil bed may be sustained because of the local activity of tissue activators, premature dissolution of local clots prolonging bleeding or provoking renewed blood-loss. It is a fair assumption to relate the haemostatic value of

15 and 30 kg 8 g EACA over 30 kg 12 g EACA. A few instances of vomiting were observed after injection of the drug

As the EACA dose had to be adapted according to the weight of the child, this part of our investigation was not a double blind but still a controlled not stratified study Each patient involved in the study was allocated to the EACA or placebo group according to serial numbering before the trial started each number was listed at random in one of the two groups. As a significant difference in blood loss was observed after treatment of 67 children (31 in the EACA group, 36 in the control group) this trial was not continued with the remaining 33 of the 100 children initially envisaged Both groups were similar with respect to weight and height. The blood-loss in the EACA treated group was 60.3 ml (SD 17) and in the control group 87.2 ml (SD 40 0 ml) this difference is highly significant ($P < 0\,001$)

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Reticulohistiocytic Origin of the Blood Basophil in Human Marrow

E. M. Schleicher

Since Ehrlich [1] described two types of mast cells the *Blutmastzelle* and the *Gewebsmastzelle* their origin and histochemistry have been studied. Because of the structural, chemical and functional similarities between the cells Selye [2] states the blood basophil represents the circulating form of the tissue mast cell system However Lennert and Schubert [3] Braunsteiner [4] Braunsteiner and Pakesch [5] Shelley and Juhlin [6] and Wetqvist [7] showed structural differences between the blood basophil and the tissue mast cell. While the genesis of the blood basophil has remained conjectural Undritz [8] derives the cell from a Basophiloblast

It is the purpose of this communication to present morphological evidence of a direct reticulohistiocytic origin of the blood basophil in human bone marrow For this study Hodgkin s disease was found to be particularly appropriate Frequently the basophils are increased in the circulating blood and the bone marrow in this condition besides, the genesis of the blood basophil could be more easily studied than in leukemic marrows containing large quantities of undifferentiated and pleomorphic cells.

Material and Method

The cell studies were made on male 28 years old, who had generalized Hodgkin dise se The peripheral blood basophils ranged from 8 to 14 determined by the method of Scott cmm [9]. From the body of the sternum 'marrow units (Markbroeckel) were separated The 'marrow unit imprint technique was employed [10, 11]. The preparations were ... ated by dual staining procedure namely Wright blood stain with Toluidin blue 0.. aqueous

solution superimposed. The latter forms chemical union with the acid mucopolysaccharides in the granules giving them the specific metachromatic ppearance.



Fig 1 Reticulohistiocytic syncytium. ( ) Primitive reticulolymphoid cell (kleine lymphoide Retikulumzelle of Rossa) (b) Early developmental stage of the blood basophil. ( ) Advanced developmental stage of the blood basophil.

Fig 2. ( ) Histiocyte. (b) Variants of developmental stages of the blood basophil. Note the cytoplasmic tail" containg various amounts of granules of different size and degree of metachromasia.

Results

In the reticulohistiocytic syncytium (fig 1) the earliest recognizable developmental stage of the blood basophil is a small reticulolymphoid cell 'kleine lymphoide Retikulumzelle' of ROHR (fig 1a) The nucleus is round and often slightly notched and more or less eccentrically located. It has a reddish-purple color and several various sized bluish-purple nucleoli. The narrow cytoplasm ranges from light blue to colorless and may contain small metachromatic granules. The cell boundary is demarcated by a condensation of the cytoplasm. This primitive cell differentiates within the syncytium into a blood basophil without going through a blast stage (fig 1b c) One to several basophils in various maturation stages may be present in a syncytium. The cells retain a histiocytic nucleus which becomes indented, lobulated and more or less overlayed by granules of different size and degrees of metachromasia. Various developmental stages are presented in fig 2b. The pear-shape results from a 'cytoplasmic tail' in which the granules tend to concentrate. This morphological feature appears to be characteristic of the developing basophil According to these morphologic findings it is inferred that the blood basophil is a histiocyte

Summary

The genesis of the blood basophil in human bone marrow as seen in marrow unit imprint preparations is presented. A direct origin from reticulohistiocytic syncytia could be demonstrated. The progenitor of the blood basophil is a small reticulolymphoid cell 'kleine lymphoide Retikulumzelle' of ROHR It is inferred that the blood basophil is a histiocyte

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Neonatal Hemolysis Due to a Transient Severity of Inherited Pyruvate Kinase Deficiency[1]

M. Bossu, M. Dachà and G. Fornaini

In recent years many works have shown the high occurrence and severity of the hemolytic diseases in the newborn period. In many cases, such as those due to an isoimmunization, to a bacterial and viral infections, to genetically determined metabolic deficiencies, to hemoglobinopathies, to morphological modifications of the cell, the etiology and pathogenesis are fairly known; in other cases, the causes of the hemolytic episode are unknown. The latter forms may be ascribed to some morphological or functional transient disturbances of the cell.

However, it might be that a transient state of deficiency is responsible for the hemolytic episode also in the subjects bearing an inherited defect and particularly in those cases in which some inborn errors of erythrocyte metabolism are present. Such a possibility is confirmed by the present case of hemolytic disease in a newborn infant being heterozygous for pyruvate kinase deficiency. In this subject the hemolytic state is determined by a transient severity of the genetic deficiency.

Case Report

M. P. Proposita. At 4 days age the patient's colouring became pallid and he demonstrated icterus, vomiting and slight splenomegaly. Examination of peripheral blood provides the

The following abbreviations have been used: *G-6-PD* glucose-6-phosphate dehydrogenase; *6-P-GD* phosphogluconate dehydrogenase; *PK* pyruvate kinase; *G-6-P* glucose-6-phosphate; *6-P-G* 6-phosphogluconate; *GSH* reduced glutathione; *APH* acetyl-phenylhydrazine; *ATP* adenosine-5-triphosphate; *RBC* red blood cells; *WBC* White blood cells; *WEP* Whole erythrocyte population.

[1] The present work has been carried out with CNR founds.

following data: RBC 2,600,000, Hb 11‰, reticulocytes 4 ‰, slight aniso-poikilocytosis, small number of macrocytes and normoblasts, no spherocytes, WBC 7,200, serum bilirubine 16.8‰. Coombs test negative. The red cell osmotic fragility was normal before incubation and markedly increased after incubation at 37°C for 24 h. The autohemolysis was not corrected by adding glucose, and slightly decreased by adding glucose and ATP. The subject was not transfused and was treated with cortisone and albumin.

24 h after crisis: RBC 2,600,000, reticulocytes 7‰, bilirubine 16.6‰; 48 h after crisis RBC 2,800,000, reticulocytes 9‰, bilirubine 15.4‰; 5 days after crisis: RBC 2,800,000, reticulocytes 14‰, bilirubine 12.4 ‰; 10 days after crisis both icterus and splenomegaly disappeared and the hand-feeding was well accepted. RBC. 3,300,000, reticulocytes 8‰, bilirubine 9.3‰; 20 days after crisis RBC 3,900,000, reticulocytes 3‰, bilirubine 5.8‰; 30 days after crisis RBC 4,200,000, reticulocytes 2‰, bilirubine 4.1 ‰.

The examinations of the subjects used as controls were normal, for the referred experiments, from a pathological point of view or from hematic tests.

Researches were always been carried out parallely on the pathological subject and on controls bearing approximately the same age.

Materials and Methods

Blood was collected with siliconated syringe and heparin added. After centrifugation the plasma and buffy coat were removed by suction and the red cells washed three times with isotonic buffered KCl solution at pH 7.4. The packed cell volume of these suspensions was determined by centrifugation at 3,000 rpm for 60 min in hematocrit tube. The hemolysates were prepared by treating washed cells with two volumes of cold distilled water. The ghosts were then removed by centrifugation at 30,000 × g for 30 min and the supernatant collected. All procedures were carried out at +2°C.

Young and old erythrocytes were prepared from resuspended washed cells according to Marks and Johnson's method [1], which is based on the principle that young erythrocytes are more resistant to hypotonic hemolysis than are older erythrocytes.

The glucose utilization by intact erythrocytes was determined according to previously described procedure [2] and the reduced glutathione stability according to Beutler [3] in presence of 5 · 10^{-3} M glucose.

G-6-PD and 6-P-GD determinations were performed according to Kornberg and Horecker [4] and Horecker and Smyrniotis [5] following Glock and McLean' suggestions [6] to remove the 6-P-GD interference on 6-P-G derived from G-6-P. Pyruvate kinase determinations were performed according to Bücher and Pfleiderer [7] and Triose P isomerase determinations according to Beisenherz [8].

Glucose was determined with glucose oxidase [9]; lactic acid according to Barker [10]; GSH according to Grunert and Phillips [11]; hemoglobin concentration with Drabkin' method [12].

In experiments of erythrocyte separation on the basis of different osmotic resistance young-cells' are those unlysed after hemolysis with 0,30% NaCl for 15 min at +2°C; old cells are those hemolysed with 0,46% NaCl under the same conditions. The number of cells was determined by hemolysis achieved by a given concentration of NaCl, taking the extent of hemolysis in water tube as equal to 100%.

In the GSH content is expressed in mg/100 ml of packed erythrocytes. The values of glucose uptake and the level of enzyme activities are referred as µmoles of metabolized substrates per hour and per ml of packed erythrocytes.

Substrates, enzymes and coenzymes were products of Sigma and Biochemica Boehringer other chemicals of Merck. Spectrophotometric determinations were carried out with Optica

CF4 spectrophotometer high speed centrifugations have been done on Lourdes LRA refrigerated centrifuge

Results

We have assayed in the erythrocytes of the propositus the GSH content and stability glucose uptake and the levels of those enzyme activities showing most frequently a genetic deficiency The results are shown in table I and compared to normal values of newborn and adult subjects. The glucose-6-phosphate dehydrogenase 6-phosphogluconate dehydrogenase and triose-phosphate isomerase activities showed normal values the GSH content is slightly decreased, but the stability test is normal as expressed in per cent values. On the contrary glucose uptake (46 , the normal values) the synthesis of lactate (48 the normal values) and the pyruvate kinase activity (18 the normal values) are markedly decreased.

These metabolic alterations and laboratory findings indicate therefore that the hemolytic episode of the propositus is due to a PK deficiency The slight decrease of GSH content my be referred to the marked lowering of glucose uptake.

We have assayed PK activity also in the young and old erythrocytes which were separated by hypotonic hemolysis. Also in both of these

Table I. Erythrocyte metabolic activities of the propositus, and of normal newborns and adults

Metabolic activities		Propositus	Normal subjects Newborn	Adults
Reduced glutathione	before APH	63	74 ± 5.8	72 4.6
	after APH	5..	61 ± 7.2	9 5.7
Glucose uptake		1.8	3.4 ± 0.4	2.6 0.5
Lactate synthesis		2.7	5.6 0.7	4.5 0.6
G-6-P dehydrogenase		73	72 ± 8.3	56 6.5
6-P-G dehydrogenase		60	63 ± 7.4	45 5.6
Pyruvate kinase		26	196 ± 25	153 ± 17
Triose-P-isomerase		4000	4,00 6.0	4900 320

The reduced glutathione content is expressed in mg/100 ml packed erythrocytes. The glucose uptake lactate synthesis and enzyme activities are referred as µmoles of metabolized substrate hour ml packed erythrocytes. The values for normal subjects are the means of literature and personal data.

fractions the enzyme activity was low being 47 units in the young cells and 19 units in the old ones.

The clinical course and the very low level of PK activity of the propositus raised the possibility of an homozygous state of the defect. Therefore we have assayed erythrocyte PK activity also in the relatives of the propositus in those cases where the enzyme activity was decreased, glucose uptake also has been determined. Table II and figure 1 show the results of the investigations performed on the relatives. The defect of PK is present in the erythrocytes of the paternal branch whereas the maternal branch is unaffected. In the affected subjects the defect appears to be in the heterozygous state the PK activity ranged between 44–70% the normal values. Glucose uptake and lactate synthesis were slowly decreased in two subjects only (case No 10 and No 16)

Table II. Erythrocyte pyruvate kinase activity and glucose utilization in the family members of the propositus

Subjects Case No.	Pyruvate kinase	Glucose utilization glucose uptake	lactate synthesis
1	84	2.7	4.8
2	168	–	–
3	153	–	–
4	108	2.5	4.3
4	138	–	–
5	174	–	–
5	142	–	
6	68	2.6	4.6
7	160	–	
8	149	–	–
9	93	2.7	4.5
10	71	2.1	3.4
11	134	–	–
12	172		–
13	165		
15	147	–	
16	87	2.0	3.1
17	159		
18	144	–	–
19	161		

The values are referred as μmoles of metabolized substrate/hour/ml packed erythrocytes.
The relationship of the reported cases is elucidated in figure 1

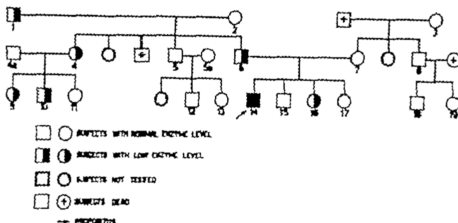


Fig 1 Pedigree of propositus family in which members were tested by the pyruvat kinase activity

As it is reported in literature PK deficiency is highly heterogeneous as concerns the activity level [13–16] Since in some heterozygous subjects the activity is much lower than in homozygous subjects, the propositus could fall within this group However it is also possible that the severity of the defect was related to the newborn state. The results reported in figure 2 are consistent with the latter hypothesis.

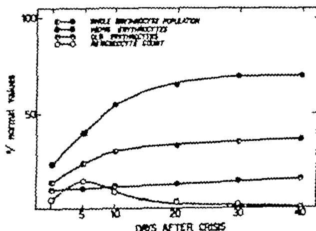


Fig 2. Pyruvat kinase activity in erythrocyte fractions of different age obtained from the propositus t different time intervals after crisis.

The erythrocyte pyruvate kinase activity of the propositus has been assayed during 40 days at different time intervals on the whole erythrocyte population and on the young and old erythrocyte fractions. The results are reported in the figure 2 as per cent of similar cell preparations obtained from newborns of the same age. In the old erythrocytes the enzyme activity is very low at every time. The enzyme from the WEP in which the reticulocyte count is very high, shows high levels of activity at the 5th and 10th day this increase is also present and progressive in the following period of observation during which blood reaches normal pictures. In the young erythrocytes the enzyme shows marked modifications with aging the activity rises rapidly during the first 20 days and then becomes stable at a level (66% the normal) which is the mean value in the heterozygous subjects.

Discussion

Many authors [17–19] have demonstrated that some transitory modifications of glucose-6-phosphate dehydrogenase induce neonatal hemolysis. Our present results show that this may be also for pyruvate kinase in the propositus the transitory state of deficiency comes out to be a greater extent of the inherited trait. The low levels of the enzyme activity could account for a homozygous state. A rather long time interval is necessary for observing the disappearance of the transitory state of the defect thus, in the young cells the enzyme level reaches values which are typical for the heterozygous state only after more than 20 days from the hemolytic episode.

Few evidences have been provided that a transitory state of deficiency affecting both normal or genetically altered enzymes, may determine hemolysis. Many reasons may account for this. The hemolysis in the neonatal period has been considered only recently from a biochemical point of view In few cases the biochemical features have been investigated during the hemolytic episode also in these cases those parameters have been examined which are usually affected by genetic mutations. In most cases, where transfusions are made, the biochemical assays are performed a long time after the crisis and therefore it is possible to detect only the metabolic inherited defects in their typical expressions.

Many authors [20–27] have incriminated some endogenous factors other than the genetic defect for the hemolytic episode in newborn

with G-6-PD deficiency The hemolysis, which is known to affect *approximately 5 % of infants bearing this defect* [27] has been shown to be independent of the degree of the deficiency moreover no known hemolytic agents could be demonstrated to determine the episode. For these cases the additional, indipendent genetic factor might be a transient state of deficiency

The mechanisms determining the transient states of deficiency are unknown. However the occurrence of this condition is probably related to the features of the newborn s erythrocyte which are very *different from those of the adult*[28] The newborn s red blood cells, besides for the HbF differ from the adult s ones as concerns electron microscopic appearance and chemical composition of the membrane, carbohydrate uptake and the activity levels of several enzymes. On this subject it must be remembered that the marked vulnerableness of newborn s cells to damage from hydrogen peroxide [29 30] and from oxidizing agents [31–35] may be ascribed to the lowered levels of glutathione peroxidase [30] and katalase [36] activities

As regards the mechanisms of hemolysis due to PK deficiency this may be framed in the general problem of that hemolysis which related to metabolic defects. It has been established that the process leading to sequestration and destruction of erythrocytes in the reticuloendothelial system, is determined by a progressive failure in energy production [37–43] and in the maintenance of the reductive state [44–49] of the cell. Both these processes are related to glucose utilization which is the only fully active metabolic process in the mature circulating cell. *Energy production occurs* by ATP resynthesis at the pyruvate kinase and phosphoglycerate kinase steps the maintenance of the reductive state is linked with reduced pyridine nucleotides synthesis through the dehydrogenase reactions of the *Embden Meyerhof Parnas pathway* and of the oxidative shunt. Thus, all the erythrocyte defects leading to modifications of either process may induce the hemolytic episode.

As concerns pyruvate kinase, when the defect is partial, that is, in the heterozygous state, *the damage is generally asymptomatic*, probably because of two reasons 1 Pyruvate kinase shows high levels of activity [41 42 50] and may work sufficiently even when it is partially damaged 2 the possible compensatory mechanism for ATP synthesis by the *Rapoport Luebering cycle* [51 52] In fact the enzymes of this cycle (phosphoglycerate kinase, diphosphoglyceromutase diphosphoglycerate phosphatase) regulate the glycolytic resynthesis of ATP The transformation of 1 3-diphosphoglycerate *into 3-phosphoglycerate may*

occur through the phosphoglycerate kinase reaction with coupled ATP resynthesis or through diphosphoglyceromutase and diphosphoglycerate phosphatase reactions with release of inorganic phosphate.

In those cases where a total defect is present the pyruvate kinase activity is so low as to block the glycolytic process, as it is clear from the decreased glucose uptake. This damage leads to a decreased ATP and pyridine nucleotides synthesis and the alterations occur (cell shape, permeability phospholipid, cholesterol and coenzyme turnover allosteric properties of Hb oxidation of Hb and of structural and enzyme proteins) determining the lysis.

Summary

A case of hemolysis in 4 days old infant is reported which is due to transient severity of heterozygous pyruvate kinase deficiency Assays performed on the whole erythrocyte population during the crisis revealed normal levels of glucose-6-phosphate dehydrogenase, *6-phosphogluconate dehydrogenase and triose-phosphate isomerase low decrease of GSH* content without any modifications of its stability marked decrease of pyruvate kinase activity (18% the normal values) and of glucose uptake (50% the normal values). The pyruvate kinase activity has been followed during 40 days from the beginning of the hemolytic episode on the whole erythrocyt population and on young and old cell fractions. It has been demonstrated that in the young cells the enzyme activity increases progressively till reaching level (66% the normal values) which is peculiar of the heterozygous state of the defect.

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Die Forschungen auf dem Gebiete der Gewebe- und Organtransplantationen machten *in den letzten Jahren* ausserordentlich grosse *Fortschritte*. Die neuen Erkenntnisse ergaben sich aus Arbeiten verschiedenster Spezialitäten, wie Immunologie, Biochemie, Genetik, Innere Medizin und Chirurgie. Es erwies sich deshalb als notwendig, dass sich die Forscher aller Spezialitäten, die an den Errungenschaften beteiligt sind, regelmässig treffen. Aus diesem Grunde fand vom *27.–30. Juni 1967* in Paris der I. Internationale Kongress der Transplantations-Gesellschaft statt. Zuerst wurden an sieben Meetings allgemeine Themen behandelt wie: Der Mechanismus der Transplantationsabstossung – Immunosuppressive Methoden – Genetische Probleme der Transplantation – Transplantationsantigene – Organtransplantation – Knochenmarktransplantation – Krebs als Transplantate. Danach folgten verschiedene Übersichtsreferate, u.a.: Betrachtungen zum Abstossungsmechanismus – Spenderauswahl für Organtransplantationen – Biologische Probleme bei Transplantationen – Immunosuppressive Substanzen. Weitere Referate befassten sich *mit* Transplantationen von Niere, Leber, Herz, Lunge, Pankreas und Gehirn.

Die im Kongressband niedergelegten Ergebnisse sind nur für den an Transplantationsproblemen speziell interessierten Mediziner bestimmt. Derjenige, der sich über *Transplantationen allgemein orientieren möchte*, sollte nicht zu diesem Buch greifen. Dem Spezialisten dagegen bietet es eine grosse Fülle von neuen Erkenntnissen.

H. T[illegible], *Basel*

Varia

VIIth International Congress of Clinical Pathology

The VIIth International Congress of Clinical Pathology will be held in Montreal, July 13th to 19th, 1969 under the joint auspices of the Canadian Association of Pathologists, the Canadian Society of Clinical Chemists and the Canadian Association of Medical Bacteriologists.

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Pathologisches Institut der Universität Köln (Direktor: Prof. Dr. M. Eder) und II. Medizinische Universitätsklinik und Poliklinik Mainz (Direktor: Prof. Dr. P. Schölmerich)

Über den Nachweis der Thiaminpyrophosphatase («Golgiphosphatase») in Blut- und Knochenmarkszellen[1]

R. Fischer und H. H. Henneberg

Novikoff und Goldfischer [17] haben eine enzymcytochemische Methode angegeben, mit der in den Zellen zahlreicher Gewebe im Bereich der Golgizone ein deutlicher Reaktionsausfall feststellbar war. Bei diesem auf dem Gomoriprinzip basierenden Nachweisverfahren dienten verschiedene Nukleosiddiphosphate sowie Thiaminpyrophosphat als Substrate, die bei annähernd neutralem pH von einer «Golgi-Phosphatase» hydrolisiert wurden. Die Annahme einer solchen spezifischen Enzymlokalisation in den lamellären und vesikulären Strukturen des Golgiapparates konnte in der Folgezeit bestätigt werden [2, 5, 13, 15, 16, 20].

Bei den vorliegenden Untersuchungen sollte das Vorkommen und die Lokalisation der Thiaminpyrophosphatase in den Zellen des Blutes und Knochenmarks geprüft werden. Die Klärung dieser Frage erschien vor allem deshalb von Interesse, weil sich gerade in jüngster Zeit wichtige Hinweise für die funktionelle Bedeutung des Golgiapparates bei der Granulabildung in den Zellen des hämatopoetischen Systems ergeben haben [3, 7, 23].

Material und Methodik

Als Untersuchungsmaterial dienten Blut- und Knochenmarksausstriche von 30 gesunden Personen bzw. von Patienten mit nichthämatologischen Erkrankungen. Außerdem wurden 20 Fälle verschiedener Leukämien untersucht.

[1] Mit Unterstützung durch die Deutsche Forschungsgemeinschaft. Auszugsweise vorgetragen auf der 12. Tagung der Deutschen Gesellschaft für Hämatologie, Berlin 1966.

Die Ausstriche wurden luftgetrocknet und anschließend fixiert. Unter den in Vorversuchen getesteten *Fixierungsmitteln* ergab die Vorbehandlung mit Formalin-Calciumchlorid oder Formalin-Calciumazetat (5 Min., 4 C) die besten Resultate.

Nach der Fixierung wurden die Ausstriche in Aqua dest. gespült und erneut luftgetrocknet.

Die *Inkubation* erfolgt 1 Std. bei 37° C in dem von Novikoff und Goldfischer [17] angegebenem Medium 20 ml 0,01 m Thiaminpyrophosphat, 8 ml Aqua dest., 40 ml 0,2 m Tris-Maleat Puffer pH 7,2 12 ml 0,03 m Bleinitrat und 20 ml 0,025 m Manganchlorid.

Nach der Inkubation wurden die Präparat mehrmals in Aqua dest. gespült, anschließend für 1–2 Min. in verdünnte Ammoniumsulfidlösung eingestellt und erneut gewässert. Die Kerngegenfärbung erfolgt mit Mayers Hämalaun. Eindecken in Glyceringelatine.

Die Spezifität der Thiaminpyrophosphatase-Aktivität wurde durch folgende *Kontrollreaktionen* geprüft a) Weglassen des Substrates oder Hitzeinaktivierung b) Zusatz von folgenden Inhibitoren zum Inkubationsgemisch 0,01 m Uranylnitrat, 0,01 m Natriumfluorid, 0,01 m Zysteinchlorid. Nach Weglassen des Substrates, Hitzeinaktivierung sowie nach Zusatz von Uranylnitrat war keine Enzymaktivität feststellbar Dagegen blieb die Aktivität der Thiaminpyrophosphatase durch die Inhibitoren der sauren Phosphatase (Natriumfluorid) und der alkalischen Phosphatase (Zysteinchlorid) unbeeinflusst.

Neben der Pappenheim-Färbung kamen an Ausstrichpräparaten der untersuchten Fälle in parallel durchgeführten Untersuchungen ausserdem folgende enzymzytochemischen Nachweisverfahren zur Anwendung: Naphthol-AS-D-Chloroacetat Esterase [14], Naphthol AS-Acetat-Esterase [12] saure Phosphatase [4], alkalische Phosphatase [11].

Ergebnisse

In der Entwicklungsreihe der *neutrophilen Granulozyten* zeigen Myeloblasten und frühe Promyelozyten nur eine geringe Aktivität der Thiaminpyrophosphatase, deren Reaktionsprodukt hier in einem kleinen umschriebenen, perinukleären Zytoplasmabezirk lokalisiert ist (Abb. 1 a) Mit der weiteren Differenzierung zu Promyelozyten fällt eine deutliche Vergrösserung der Aktivitätszone auf die relativ scharf begrenzt in der Nähe der beginnenden Kerneinbuchtung liegt (Abb 1 b und c) Das Reaktionsprodukt zeigt in den Promyelozyten häufig eine ring bzw schalenförmige Anordnung deren Mitte etwa der Lage der Zentriolen entspricht (Abb 1 b) Auch im Myelozytenstadium findet sich zumeist eine deutliche Fermentaktivität, deren Ausdehnung im Vergleich zu den Promyelozyten etwas abgenommen hat (Abb 1 d und e) Noch deutlicher wird die Abnahme der Enzymaktivität bzw die Verkleinerung der Reaktionszone mit der weiteren Ausreifung der neutrophilen Granulozyten. Während in den Metamyelozyten häufig noch einzelne Bleisulfidniederschläge zu beobachten sind (Abb 1 f) war in den stab- und segmentkernigen Neutrophilen in der Regel keine sichere Enzymaktivität mehr nachweisbar

Die Aktivitätszone der Thiaminpyrophosphatase in den Promyelozyten entspricht der im panoptisch gefärbten Präpara erkennbaren Lokalisation der beginnenden Azurgranulo-



a, b,



d, f



g, h,

Abb. 1. Nachweis der *Thiaminpyrophosphatase* in Zellen des menschlichen Knochenmarks) Myeloblast, b) und) Promyelocyten, d) und) Myelocyten, f) Metamyelocyt, g) eosinophiler Myelocyt, h) und i) Plasmazellen. Relativ scharf lokalisierte Reaktion in einem perinukleären, der Golgizone entsprechenden Bezirk. × 1400.

tion. Eine ähnliche, zunächst auf einen perinukleären Bezirk beschränkte Reaktion zeigt im frühen Promyelocyten auch der zytochemische Nachweis der *Naphthol-AS-D-Chloracetat-Esterase* (Abb. 2) oder anderer an die Leukocytengranula gebundener Enzyme (Peroxydase, Naphthol-AS-Acetat-Esterase, saure Phosphatase).

Der Reaktionsausfall in den *eosinophilen Granulocyten* stimmt im Prinzip mit den geschilderten Befunden an den Zellen der neutrophilen Entwicklungsreihe überein. Im allgemeinen fällt der Nachweis der Thiaminpyrophosphatase in den Eosinophilen jedoch etwas kräftiger aus als in den entsprechenden neutrophilen Zellen. Besonders deutlich tritt die charakteristische zirkuläre Orientierung der Aktivitätszone in den eosinophilen Promyelocyten und Myelocyten hervor



Abb. 2. Darstellung der *Naphthol-AS-D-chloracetat-Esterase* in frühen Promyelozyten mit einem zunächst auf einen perinukleären Bezirk (Golgizone?) beschränkten Reaktionsprodukt (a und b), *das sich bei der weiteren Differenzierung der Zellen* auf größere Abschnitte ausdehnt (c) und schließlich das Zytoplasma völlig ausfüllt (d). Promyelozyten-Leukämie. × 1400.

(Abb. 1 g). Außerdem war im Gegensatz zu den enzymnegativen stab- *und segmentkernigen Neutrophilen* auch *in den reifen eosinophilen* Leukozyten gelegentlich noch eine geringe Aktivität der Thiaminpyrophosphatase in Form einzelner punktförmiger Granula zwischen den beiden Kernsegmenten nachweisbar.

Durch eine besonders starke Thiaminpyrophosphatase-Aktivität fallen die in den Knochenmarkausstrichen nachweisbaren *Megakaryozyten* auf. Das Reaktionsprodukt ist in den Vorstufen mit noch unsegmentiertem Zellkern in einer deutlich ausgeprägten perinukleären Zone lokalisiert (Abb. 3a). Besonders bei stärkerer Vergrößerung ist hier eine lamelläre und granuläre Anordnung feststellbar. Mit zunehmender Kernlappung und Ausdifferenzierung der Megakaryozyten dehnt sich die Aktivitätszone über grössere Anteile des Zytoplasmas aus (Abb. 3b und c). Neben unregelmäßigen Verdichtungen des Reaktionsproduktes lassen sich netzförmige Strukturen oder granuläre bzw. feinvakuoläre Reaktionsniederschläge in kettenförmiger Anordnung feststellen.

Sowohl in den Zellen der *Erythrozytopoese* als auch in den *Lymphozyten* im peripheren *Blut* und Knochenmark war mit der von uns ange-



Abb. 3. Deutliche Aktivität der *Thiaminpyrophosphatase* in Megakaryocyten. Das Reaktionsprodukt ist in einer perinukleären Zone ( ) mit zunehmender Kernlappung in einem größeren Zytoplasmabezirk (b) z.T. in netzförmiger bzw. feinvakuolärer Anordnung ( ) erkennbar × 900

wandten Technik kein sicherer Reaktionsausfall der Thiamin-pyrophosphatase nachweisbar. Eine zumeist deutliche Enzymaktivität

in einer umschriebenen, etwa der perinukleären Aufhellung entsprechenden Zone fand sich dagegen in den *Plasmazellen* des Knochenmarks (Abb 1h und i) *Monozyten* des peripheren Blutes zeigten eine insgesamt schwache Reaktion im Bereich der Kerneinbuchtung

Bei den untersuchten *Leukosen* entsprach die Aktivitätsverteilung der Thiaminpyrophosphatase weitgehend dem Differenzierungsgrad der Zellen So zeigten unreifzellige myeloische Leukämien je nach ihrer Differenzierungstendenz zu promyelozytären Zellen eine unterschiedlich entwickelte Golgiphosphataseaktivität. Auch *in den neutrophilen* und eosinophilen Promyelozyten und Myelozyten der chronischen Myelose bestand, ähnlich wie in den entsprechenden Zellen des normalen Knochenmarks, eine deutliche Enzymaktivität. Die Zellen lymphatischer Leukämien wiesen keine zytochemisch nachweisbare Aktivität der Thiaminpyrophosphatase auf.

Diskussion

In der seit langem geführten Diskussion über die Existenz und die funktionelle Bedeutung des in enger struktureller und funktioneller Beziehung mit dem endoplasmatischen Retikulum stehenden Golgiapparates konnte inzwischen eine weitgehende Klärung herbeigeführt werden [Übersichten 9 und 21] Vor allem die neueren Ergebnisse der Elektronenmikroskopie haben gezeigt, dass Strukturen des Golgisystems, allerdings in sehr unterschiedlicher Ausprägung eine obligatorische Komponente der tierischen Zellen darstellen. Zur lichtmikroskopischen Darstellung des Golgiapparates hat sich in jüngster Zeit besonders der von Novikoff und Goldfischer [17] eingeführte zytochemische Nachweis einer Nukleosiddiphosphate bzw Thiaminpyrophosphat hydrolysierenden Phosphatase bewährt, deren spezifische Lokalisation in Strukturen des Golgisystems inzwischen durch zahlreiche licht und elektronenmikroskopische Untersuchungen an verschiedenen Geweben bestätigt worden ist [2 5 13 15 16 20]

Die vorliegenden Ergebnisse zeigen dass die zytochemische Lokalisation der Thiaminpyrophosphatase auch in den *Zellen* des hämatopoetischen Systems mit der aus elektronenmikroskopischen Untersuchungen bekannten Anordnung des Golgiapparates völlig übereinstimmt. In der neutrophilen Granulozytopoese findet sich das Maximum der Enzymaktivität im Promyelozyten und Myelozytenstadium

während in den reifen Neutrophilen mit der angewandten Methode keine nennenswerte Reaktion mehr nachweisbar war. Auch elektronenmikroskopisch zeigt der in Myeloblasten nur spärlich ausgebildete Golgiapparat in den Promyelozyten und Myelozyten eine deutliche Vergrösserung, um sich mit der weiteren Ausreifung bis auf einige rudimentäre Zysternen wieder zurückzubilden [1, 3, 6, 22]. Entsprechende Übereinstimmungen ergeben sich auch in der eosinophilen Entwicklungsreihe.

Der besonderen Ausprägung des Golgiapparates im Promyelozyten- und Myelozytenstadium muss in Übereinstimmung mit den allgemeinen Vorstellungen über die funktionelle Bedeutung dieses Zellorganells *eine* wichtige Rolle bei der Granulabildung zugesprochen werden, die in enger räumlicher Beziehung zu Strukturen des Golgikomplexes steht [1, 3, 7, 23]. So entwickeln sich beispielsweise in den neutrophilen Vorstufen im Knochenmark des Kaninchens die azurophilen Granula durch Ausstülpung und nachfolgende Aggregation von Mikrobläschen an der konkaven, d. h. der dem Zentriol zugewandten Fläche des Golgiapparates, während die im Myelozytenstadium auftretenden «spezifischen» Granula durch Abschnürung und Konfluenz von Golgibläschen an der konvexen Begrenzung entstehen [3]. In *Übereinstimmung mit diesen elektronenmikroskopischen Befunden* stehen die Ergebnisse autoradiographischer Untersuchungen, bei denen nach Inkorporation von H Lysin der intrazelluläre Fluss der markierten Aminosäure von Strukturen des endoplasmatischen Retikulums über den Golgikomplex in die neugebildeten Granula verfolgt werden konnte [7].

Tatsächlich lässt sich auch lichtmikroskopisch in frühen Promyelozyten im Bereich der zytochemisch darstellbaren Golgiphosphatase nicht nur im panoptisch gefärbten Präparat der Beginn einer azurophilen Granulation nachweisen, sondern auch eine zunächst auf diese Zone beschränkte Aktivität bestimmter an die Leukozytengranula gebundener Enzyme wie der Peroxydase oder der Naphthol-AS-D-Chloroazetat Esterase (Abb. 2) feststellen. Eine ähnliche Lokalisation der sauren Phosphatase und anderer hydrolytischer Enzyme [1, 23] liefert zudem einen weiteren Beleg für die Lysosomennatur der Leukozytengranula sowie Hinweise für die Bedeutung des Golgikomplexes bei der Entstehung «primärer» Lysosomen überhaupt. Dabei ist die saure Phosphatase jedoch im Gegensatz zu der Nukleosiddiphosphate oder Thiaminpyrophosphat hydrolisierenden «Golgiphosphatase» nicht in den lamellären Strukturen des Golgiapparates selbst lokalisiert

sondern in den benachbarten lysosomalen Granula anzutreffen [16, 17].

Eine besonders auffällige Aktivität und Verteilung der Thiaminpyrophosphatase liess sich bei unseren Untersuchungen in den Megakaryozyten des Knochenmarks feststellen. Sie entspricht auch hier den elektronenmikroskopischen Befunden eines sehr stark ausgeprägten Golgiapparates, der bereits in den Megakaryoblasten im perinukleären Zytoplasma eine grosse, aus mehreren herdförmigen glatten Membranen und vielen Mikrobläschen aufgebaute Zone bildet. Unter wesentlicher Beteiligung des Golgiapparates entstehen in den Megakaryozyten aus Eiweissbausteinen der Grundsubstanz die verschiedenen Vorstufen des Granulomers und der Demarkationsbläschen der Thrombozyten [10, 18, 19].

Auch in den Zellen des hämatopoetischen Systems kommt somit dem Golgiapparat eine wesentliche Bedeutung im Rahmen «sekretorischer» Leistungen zu, wie sie etwa von den Plasmazellen oder Geweben mit einer starken Eiweissynthese bereits seit längerem bekannt ist. Durch die Thiaminpyrophosphatase-Reaktion ergibt sich nicht nur die Möglichkeit, die Struktur des Golgiapparates in den Zellen des Blutes und Knochenmarks mit einer den klassischen Imprägnationsverfahren überlegenen enzymzytochemischen Methode darzustellen, sondern auch seine Variabilität unter funktionellen Bedingungen, wie etwa bei den Transformationsvorgängen der Blutlymphozyten *in vitro* [8], zu erfassen. Vor allem durch die Kombination mit anderen Enzymnachweisen werden darüber hinaus auch auf zytochemischer Basis weitere Hinweise auf die Bedeutung des Golgikomplexes bei der zellulären Differenzierung der blutbildenden Systeme möglich sein.

Zusammenfassung

Der zytochemische Nachweis der Thiaminpyrophosphatase in Zellen des Knochenmarks zeigt eine unterschiedlich starke Enzymaktivität, deren Lokalisation mit der im elektronenmikroskopischen Untersuchungen bekannten Anordnung und Grösse des Golgiapparates übereinstimmt. In der neutrophilen und eosinophilen Entwicklungsreihe zeigen Promyelozyten und Myelozyten das Maximum der Enzymaktivität. Durch eine besonders ausgeprägte Reaktion der Golgiphosphatase sind die Megakaryozyten gekennzeichnet. Ausserdem ist eine zumeist deutliche Enzymaktivität in den Plasmazellen feststellbar. Der Thiaminpyrophosphatase und der besonderen Ausprägung des Golgiapparates in bestimmten Knochenmarkszellen muss eine wichtige Rolle bei den sekretorischen Leistungen dieser Zellen, vor allem bei der Granulabildung, zugesprochen werden.

Summary

Cytochemical demonstration of thiamine pyrophosphatase in bone marrow cells reveals different degrees of enzyme activity according to the arrangement and size of the Golgi apparatus known from electron microscope studies. Among the neutrophils and eosinophils, maximum activity is found in the promyelocytes and myelocytes. Megakaryocytes are characterized by particularly marked Golgi phosphatase reaction. Fairly marked activity is also generally seen in the plasma cells. The presence of thiamine phosphatase and the particular development of the Golgi apparatus in certain bone marrow cells must play an important part in the secretory activity of these cells, especially formation of the granula.

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Serum Lactic Dehydrogenase Activity in Refractory Anemia[1]

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Serum lactic dehydrogenase activity (LDH) is markedly elevated in patients with untreated megaloblastic anemias secondary to folic acid or Vitamin B_{12} deficiency and this activity rapidly falls with appropriate therapy [1–4]. The serum LDH levels in the anemias of iron deficiency and bone marrow aplasia are normal, but levels in hemolytic anemias are frequently elevated [3]. Over the past several years we have been following a group of patients with refractory anemias, some with ineffective erythropoiesis and demonstrating sideroblasts, others presenting with Di Guglielmo's syndrome. It is often difficult to distinguish morphologically the megaloblastoid changes in these cases from that noted in pernicious anemia or folate (FA) deficiency. In this paper results of studies on the behavior of serum LDH and LDH isozymes will be reported.

Methods and Materials

There were 9 patients in this series, all characterized by severe anemia, low reticulocytes, variable red cell morphology in the peripheral blood and bone marrow showing marked erythroid activity with reversal of the myeloid to erythroid ratio. Red cells were of varying degrees of immaturity ranging from pronormoblasts with normal maturation to a population of primitive erythroid precursors associated with multi-nucleated dysplastic normoblasts. Megaloblastic like changes were frequently noted. In 7 of the 9 cases Prussian Blue stain demonstrated ringed sideroblasts and generally increased iron deposition in phagocytic histiocytes. In some cases there was an increased number of promyelocytes and myelocytes, but striking absence of macrometamyelocytes and other myeloid changes usually associa-

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ted with B_{12} or folate deficiency Over several months, 3 patients progressed to overt acute granulocytic leukemia and expired. Six patients were refractory to therapy two of which required frequent transfusions (table I) Serum LDH activity was measured by the methods of WROBLEWSKI [3] and the percentage of serum LDH isoenzyme ('Heart' type) by the method of DAWSON *et al.* [6].

Results

There were no significant differences in the LDH levels or the percent of LDH isozymes in the serum of a group of 15 normal controls compared to the patients with refractory anemia (fig 1) The mean serum LDH level for normals was 221 5 units and for the patients 241 units.

Discussion

The extremely high serum LDH level reported in megaloblastic anemias associated with B_{12} and FA deficiency was not present in patients with refractory anemia characterized by increased iron and ringed sideroblasts. These findings are in agreement with observations made by HOFFBRAND *et al.* [7] The elevated serum LDH activity in megaloblastosis has been presumed to come from either increased hemolysis or directly from cell breakdown in the bone marrow [2, 3]

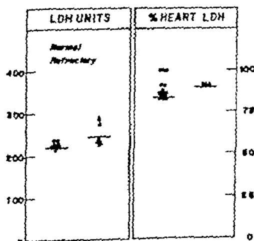


Fig 1 Serum LDH and LDH isoenzyme activity in 9 patients with refractory anemia and 15 normal controls.

Table I Clinical and laboratory data in nine cases of refractory anemia

Case No	Age/Sex	L/D	1st seen	Death	Hb g%	Hct %	WBC	Retic %	Bone marrow	Folic acid ng	B_{12} pg	Fe µg	LDH w.u	H %
1	67 ♂	+	11/30/64		7.5	24	2850	1.4	Ringed siderobl.	300	849	197	277	91
2	72 ♀	+	12/23/63		9.8	25	8800	2.2	Ringed siderobl.	3.3	689	120	312	91
3	74 ♂	+	6/28/66		8.2	25	3000	2.2	Ringed siderobl.	225	671	224	222	68
4	80 ♂	—	7/1/66	10/13/68	8.5	27	7200	1.8	Ringed siderobl.	30	1823	–	340	–
5	56 ♀	+	10/31/66		7.5	25	3300	2.3	Ringed siderobl.	17.2	681	249	228	84
6	67 ♂	+	11/30/66		8.8	26	4000	0.4	Ringed siderobl.	3.6	383	100	142	91
7	42 ♀	—	12/28/66	5/31/67	10.0	32	1450	3.0	Incr F	–	–	171	225	75
8	43 ♀	—	1/10/67	5/19/67	6.0	19	2000	1.8	Incr F	11.9	745	117	241	100
9	48 ♂	+	9/13/67		9.6	28	7000	3.2	Ringed siderobl.	40	2080	172	290	–
									Normal values	7–15.9	200–900	50–150	221.5	84

+ living, – dead
Converted to acute granulocytic leukemia
Wroblewski units

This increase has been attributed to an absolute increase in LDH_1 and LDH_2 the two elektrophoretically fast LDH isoenzymes [3] found in adult red cells. In the refractory anemias there are probably two defects an 'ineffective' type of erythropoiesis of marked degree and hemolysis resulting from increased destruction of defective red cells in the bone marrow [8]. The normal serum LDH levels in these 9 patients is of diagnostic importance and raised the question as to the source of elevated LDH activity in megaloblastic anemias.

Our observations suggest that the ineffective erythropoiesis in cases of refractory anemia may not be due to abnormal cellular proliferation, but rather to an accumulation of immature red cells with decreased destruction within the bone marrow. On the other hand, the ineffective erythropoiesis in megaloblastosis may be a result of rapid destruction of immature red cells with release of LDH from the marrow. Recent evidence of 'ineffective granulocytopoiesis' raises the alternative possibility that much of the elevated serum LDH activity may be derived from the myeloid elements in the megloblastic bone marrow [9, 10].

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Summary

Normal serum levels of LDH and its isoenzymes were found in 9 cases of refractory anemia. The red cell activity and morphology in these cases was often indistinguishable from that seen in pernicious anemia. The source of elevated LDH activity in megaloblastosis due to B_{12} and folic acid deficiency may therefore not be derived from the increased erythroid activity but rather the increased myeloid elements which are not present in refractory anemia.

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Cytogenetic Studies in Acute Leukemia[1]

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The discovery of a specific chromosomal abnormality in chronic myeloid leukemia [1, 7, 8] has led to a revival of interest in the karyotyping of human tumors. In this respect the acute leukemias have received special attention, at least partly because of the difficulties frequently encountered in classifying them on cytological grounds alone.

The present report deals with the results of cytogenetic studies on bone marrow aspirates from 7 consecutive patients admitted to the American University Hospital with the diagnosis of acute leukemia.

Materials and Methods

Six patients were males and one was female. The age range was 16-63 years, with only one patient (LCP-3) above the age of 45 years. Four had received no treatment prior to karyotyping; one (LCP 14) had prednisolone for one month, another (LCP 18) had nitrogen mustard for 7 days, 2 months prior to the examination, and a third patient (LCP-7), the only female in the group, was on both prednisolone and 6-mercaptopurine at the time of the investigation.

As to the cytological type, 3 of the patients had myeloblastic, three lymphoblastic and one monoblastic leukemia. The criteria used in differentiating these types have been described in detail by Hayhoe *et al.* [3]. Features of diagnostic importance were cellular and nuclear morphology, nuclear-cytoplasmic ratio, the presence of certain cytoplasmic inclusions such as Auer rods, as well as cytochemical reactions of the leukemic cells.

Sternal bone marrow aspiration was performed on every patient at the time of admission. A small portion of the aspirate was used for cytological examination following coloration with the Romanowsky stains, and for cytochemical reactions [3]. The rest was utilized in the

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cytogenetic studies using a slight modification of the method of Tjio and Whang [11], without prior in vitro culturing (direct marrow preparations). At the same time peripheral leucocyte counts were made and smears prepared for differential counts and for cytochemical studies.

Table I contains the pertinent clinical and hematological data.

Results

The results of the chromosome studies are shown in table II. In 4 of the patients all available plates were suitable for analysis, and all showed an entirely normal chromosome constitution. In the fifth patient (LCP 18) most of the plates were found to be normal, only 3 were hypodiploid, with a random loss of one or more chromosomes.

Distinct abnormalities were present in the marrow aspirates from 2 patients. The first, LCP 3, an 83-year-old male with myeloblastic leukemia, had not received any treatment prior to the study. Smears from his bone marrow showed numerous pleomorphic blasts with frequent mitoses, and several macroblasts and binucleate cells. Very few megakaryocytes could be seen. Cytogenetic studies revealed the presence of 2 cell-lines: a major line with a chromosome count of 41 (fig. 1) and a minor near-tetraploid line of cells with 82 chromosomes. None of these latter were of adequate quality for accurate analysis.

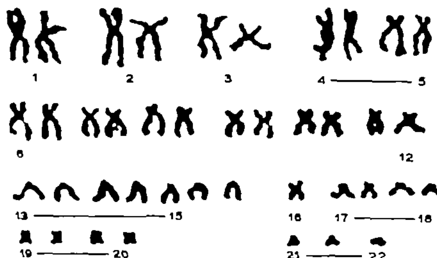


Fig. 1. Case LCP-3. Chromosome constitution of hypodiploid bone marrow cell. Note missing Y chromosome, extra D and deficiencies in groups C, E and G.

Table 1 Clinical and hematological data

Patient	Age years	Sex	Duration of symptoms prior to admission	Treatment prior to karyotyping	Leucocyte count	Peripheral blood	Sternal arrow
LCP-3 Case 218158	83	M	3 weeks	None	22,000	Myeloblasts: 75% Promyelocytes: 3% Myelocytes: 4% Metamyelo.: 4%	Numerous pleomorphic blast cells. Mitoses frequent A number of 'macroblasts and binucleate blast-like cells. Erythropoiesis markedly depressed. Very few megakaryocytes.
LCP-8 Case 221809	19	M	4 weeks	None	104,000	Lymphoblasts: 82% Lymphocytes: 9%	Numerous lymphoblasts and immature lymphocytes. Granulopoiesis and erythropoiesis depressed.
LCP 7 Case 200819	30	F	8 months	6-mercaptopurine prednisolone	8,550	Myeloblasts: 81% Promyelocytes: 1% Myelocytes: 1%	Numerous pleomorphic blasts, some having Auer bodies. Mitoses frequent Erythropoiesis relatively active normoblastic. Lymphocytes common.

Table I (continued)

Patient	Age years	Sex	Duration of symptoms prior to admission	Treatment prior t karyotyping	Leucocyte count	Peripheral blood	Sternal arrow
LCP-8 Case 222744	43	M	6 months	Vitamins Penicillin-Streptomycin	31,000	Myeloblasts: 80% Promyelocytes: 1% Myelocytes 1%	Numerous blast cells, many having azurophilic granules. Mitoses occasional. Erythropoiesis markedly depressed
LCP 13 Case 223939	16	M	1 week	None	210,000	All cells classified as lymphoblasts	Numerous lymphoblasts and immature lymphocytes. Granulopoiesis and erythropoiesis markedly depressed.
LCP 14 Case 226010	26	M	3 months	Prednisolone 1 month	2,300	Monoblasts and Promonocytes: 81% Neutrophils: 18%	Numerous monoblasts, promonocytes and monocytes. Granulopoiesis markedly depressed. Erythropoiesis relatively active
LCP 18 Case 229125	23	M	3 months	Nitrogen mustard 7 days, 2 months earlier	1,950	Lymphoblasts: 70% Lymphocytes 25%	Numerous lymphoblasts. Mitoses few Granulopoiesis relatively active. Erythropoiesis depressed.

Table II Results of chromosome studies on sternal marrow aspirates

Patient	Diagnosis	Total cells analysed	Chromosome count 40	41	44	45	46	polyploid	Marker chromosome	Remarks
LCP-3	AML	17		13				4 (near tetraploid)	–	Two abnormal cell lines
LCP-6	ALL	12					12		–	Normal karyotype
LCP 7	AML	17				7	10		? No. 11	Two abnormal cell lines
LCP-8	AML	14					14		–	Normal karyotype
LCP 13	ALL	5					5		–	Normal karyotype
LCP 14	A. monocytic L.	10					10		–	Normal karyotype
LCP-18	ALL	12	1		1	1	9		–	

Normal karyotype. Missing 1C, Missing 1 No.2 1 No.17 Missing 1C, 1D 1 No.16, 2F Y
ALL Acut lymphoid leukemia, *AML* Acut myeloid leukemia.

The second patient, LCP 7 was a 30-year-old female with myeloblastic leukemia. Marrow smears showed numerous pleomorphic blasts, some with Auer bodies. Mitoses were frequent among them. Cytogenetic studies showed two abnormal cell-lines, one with 46 chromosomes and the second with a count of 45 Examples of both lines are shown in figures 2 and 3

Discussion

To date, several hundred cases of acute leukemia have been studied cytogenetically In some the studies were performed on direct bone marrow preparations in others peripheral blood leukocyte cultures were used alone or in combination with the bone marrow preparations. The advantages of direct marrow methods over leucocyte cultures have been stressed by HUNGERFORD *et al.* [4] and more recently by KROGH JENSEN [6] Although marrow specimens in the absence of *in vitro* culturing often yielded fewer mitoses for analysis, the culturing of peripheral leucocytes has frequently resulted in the loss of abnormal cell lines, or in the preferential growth of some cell lines over others. Or if phytohemagglutinin was used in the culture, this stimulated normal lymphocytes to proliferate and predominate the cultured cell population.

Even where no culture methods were used, a variable number of patients with acute leukemia were found to have no demonstrable chromosomal aberrations. In the remaining cases, various abnormalities have been encountered. The overall incidence of these as reported in the world literature was approximately 60% [2] although REISMAN *et al.* [9] were able to demonstrate abnormal cell lines in all specimens studied during the active phase of the disease in children. In our present series only 2 of the 7 patients had karyotype changes in their marrow cells.

The chromosome groups most frequently affected were groups C and G and less commonly groups D E and F Both of our cases had deficiencies in group G and an extra member in group D In addition, case LCP 3 had deficiencies in groups C and E, and one of the lines of case LCP 7 had a missing E.

In contrast with the specific abnormality present in chronic myeloid leukemia, no characteristic chromosome pattern has been established for any type of acute leukemia. In general, it has been found that in lymphoblastic leukemia the abnormal cell lines are usually hyper



Fig. 2. Case LCP 7. Chromosome constitution of the cell line with 46 chromosomes. Note abnormally short? No. 11 deficiency in group G, and extra D.

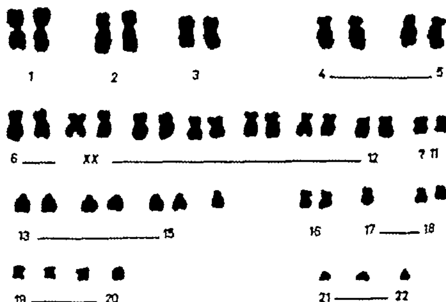


Fig. 3. Case LCP 7. Chromosome constitution of hypodiploid line. Pattern as in figure 2, but in addition, member of pair No. 17 is missing.

diploid, while in the acute myeloid type the abnormal cells are often hypodiploid. An exception to this is the small group of elderly patients with acute myeloid leukemia in whom SANDBERG *et al.* [10] found 2 abnormal lines one hypodiploid and the other hyperdiploid. Our case LCP 3 appears to have many features in common with this group

In conclusion, despite the small size of the series studied our findings are in agreement with the reports in the literature, namely that cytogenetic abnormalities are not demonstrable in many cases of acute leukemia, and if present, they are not of specific diagnostic significance.

Summary

Cytogenetic studies are reported on direct bone marrow preparations from 7 patients with acute leukemia. Abnormalities were present in 2 patients with myeloblastic leukemia, one of whom was receiving cytotoxic drug therapy at the time of the examination. The remaining patients, including 3 with lymphoblastic leukemia, had no demonstrable chromosome changes. The above findings are compared with results of similar investigations carried out elsewhere.

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A Trial of Slow Release Ferrous Sulphate (Ferrogradumet®) in Prevention of Iron Deficiency in Pregnancy

R. W. BURSLEM, L. POLLER and H. WACKS

We have attempted to assess the effectiveness of a slow release ferrous sulphate tablet given once daily as opposed to conventional ferrous sulphate given three times daily in the prevention of anaemia.

An ideal situation for the study of such preparations arises during pregnancy which results in iron deficiency in a considerable proportion of patients when prophylactic iron is not administered. A controlled *trial has, therefore, been performed of slow release ferrous sulphate* with folic acid supplement, studied in parallel with a conventional ferrous sulphate tablet and folic acid. A large number of ante natal patients attending a general maternity hospital has been followed.

Method

The effectiveness of the slow release iron in the prevention of anaemia was evaluated by means of two large parallel groups of ante-natal patients. It was found advisable to use alternate clinics for the selection of patients for the two groups. Two booking clinics every week are held at this hospital. They were identical in all respects.

The series covered initially all patients attending the booking clinic for the first time over a three-month period (September 1963–December 1963). Details were recorded of age, parity, gravidity and previous illnesses. Initial tests included haemoglobin, examination of a blood film and urine bacteriology.

Routinely haemoglobin readings are expressed grams per cent. For convenience in the analysis percentage haemoglobin figures were used 100% = 14.6 g with the conventional cyanmethaemoglobin standard.

All patients were given 3 months supply of slow release ferrous sulphate (Ferrogradumet) and folic acid, or combined conventional ferrous sulphate/folic acid. The former group took one tablet daily containing 105 mg elemental iron and one 5 mg folic acid tablet, and the latter group took one tablet three times daily giving a total of 180 mg elemental iron daily and an equivalent dose of folic acid.

Normal patients were not seen again until 30 weeks in the ante-natal clinic following general practitioner care.

From the 30th week of gestation onwards each patient had regular haemoglobin check (capillary finger prick cyanmethaemoglobin) at least once every 4 weeks, although in some cases the haemoglobin was repeated weekly or fortnightly.

If the haemoglobin was below 10.2 g (70%) venous specimen was taken for absolute values. If the venous haemoblogin was found to be less than 10.2 g routine serum iron, B_{12} and folate were advised. I some cases marrow aspiration was performed.

All patients had haemoglobin estimation just prior to delivery and, in nearly all cases within 48 h *post partum*. During the ante-natal period, not was taken of urinary infection, toxaemia, bleeding, hyperemesis and various complications as well as the number of abortions, stillbirths, live births, premature and multiple births and perinatal deaths.

Finally the need for parenteral iron *pre* or *post partum* or any change in the regime of slow release ferrous sulphate to any other form of iron was noted.

Subjective data were not included, e.g. whether the patients said they were taking their tablets, whether they suffered nausea or constipation. It was assumed that with such large numbers this would be reflected in the haemoglobin level.

Results

Of 472 patients in the trial, 228 received slow release ferrous sulphate and folic acid and 224 were given folic acid and ferrous sulphate in conventional combined form. The mean parity of the slow release group was 1.5 and 1 6 with the conventional group The mean age was 26 years in both groups.

Ten patients were lost from each group because of confinement at another hospital, abortions, etc. The percentage of patients given parenteral iron was identical at (11 4%) in both groups. Nine addition al patients were lost from the slow release iron group because they were changed to other oral iron preparations at some stage during the trial.

The effect on haemoglobin levels was as follows. The mean haemogloban figure after delivery was 79.3% for the slow release groups and 78.7% for the other group. The weekly mean haemoglobin levels are seen in table I Patients who were anaemic had more frequent estima tions of their haemoglobin their results were analysed separately (table II) There were 54 patients with initial haemoglobin of less than 10.2 g in the slow release ferrous sulphate series and 60 patients in the conventional series. Their mean haemoglobins at delivery were 81 1% and 80.3% respectively There was no significant difference in the speed of response of anaemic patients to the two preparations when analysed on a weekly basis using students 't test' (table II) The gross incidence of anaemia in the two groups seemed to be very close and there was no significant difference between the groups.

Table I Mean Hb levels, %

Weeks	14	28	29	30	31	32	33	34	35	36	37	38	39	40	*Post partum*
Slow release ferrous sulphat	79.4	76.3	78.9	78.0	77.9	78.5	78.12	78.7	80.2	80.2	80.4	81.7	81.7	81.2	79.3
						Mean of 37th–40th week levels = 81.3%									
Ferrous sulphate folic acid	82.6	78.6	79.9	77.9	79.2	77.9	79.3	79.3	81.5	80.4	80.1	80.3	81.5	80.5	78.7
						Mean of 37th–40th week levels = 80.6%									

Table II Mean Hb levels (%) of patients with 10.2 g and under at initial reading (excluding patients given parenteral iron)

Weeks	Initial	28	29	30	31	32	33	34	35	36	37	38	39	40	*Post partum*
Slow release ferrous sulphat	69.5	70.9	78.5	77.8	78.1	74.9	75.3	78.2	77.6	78.9	79.6	79.7	80.5	79.1	80.6
Ferrous sulphat folic acid	68.2	71.0	72.3	72.4	72.2	72.7	74.7	73.0	73.9	77.3	76.3	76.4	78.9	76.3	79.6

The incidence of complications of pregnancy was as follows. In the slow release iron group urinary infections 19 (8.3%) antepartum haemorrhage 2, hyperemesis 1 in the conventional iron group urinary infections 17 (6.9%) antepartum haemorrhage 4 hyperemesis 1 Premature births were 7 and 6 respectively and the number of ante-natal admissions for various causes was 38 in the slow release iron, and 45 in the other group

Discussion

At the start of the trial the two groups of patients appeared identical. Their ages and parity were similar During the trial obstetrical complications, were similar There was no increase in the number of premature births, abortions or stillbirths. Ante-natal admission rates were similar in the two groups and the number of patients who had hospital admission for investigation of anaemia was insignificant.

The incidence of non-responsive anaemia in patients treated with prophylactic iron and folic acid is of interest. In both series this was 11.4% There is thus a hard core of cases in whom anaemia was not prevented by a prophylactic regime. Similar figures have been reported in the literature. HENDERSON [2] found that regardless of the combination of oral agent used approximately 10% of patients remained anaemic. CHISHOLM [1] in a survey in Oxford found an incidence of anaemic of 12% in spite of prophylactic iron therapy

It appears that the one tablet daily regime of the administration of slow release iron is as effective clinically as giving iron three times daily in conventional form. As the amount of elemental iron is less in the slow release tablets it must be assumed that absorption is more efficient. It does not appear however that this regime is significantly better clinically in the prevention of anaemia.

If the patients acceptability of the new preparation had been superior to conventional iron it might be assumed that the mean haemoglobin would have been maintained at a higher level or that the incidence of anaemia would have been less due to the elimination of cases unable to tolerate conventional ferrous sulphate.

The analysis of the anaemic cases showed that the two preparations were equivalent in their long term effects, no significant difference being found in the two groups at term. Although the differences were not significant the results do perhaps suggest that the response to slow release ferrous sulphate may have been more rapid and this is also

suggested by the higher number of anaemic patients in the conventional ferrous sulphate group who required parenteral iron. Similar accelerated response has been suggested by Israëls and Cook [3] in the treatment of established anaemia. This trial however was not designed to assess the speed of response of established anaemias.

Conventional ferrous sulphate is used on a large scale in hospitals in this country and is consequently extremely cheap. The cost of a combined conventional ferrous sulphate/folic acid tablet for a 28 week course per patient is 4/6d. The preparations of slow release ferrous sulphate (Ferrogradumet®) used in this study is much more expensive, the current cost for a 28 week course at the best hospital price being over 30/ per patient. There might be a case for routine use of the slow release ferrous sulphate if substantial benefit could be shown. In the present study we have found no significant benefit in the prevention of anaemia in pregnancy as distinct from the treatment of established anaemias.

Acknowledgment

We are grateful to Abbott Laboratories for some statistical assistance with the trial and for supplies of Ferrogradumet® and Dr. A. Anthony, Resident Clinical Pathologist, for help in the early stages of the study.

Summary

A trial of the effectiveness of a slow release, once daily preparation of ferrous sulphate has been performed in the prevention of anaemia in pregnancy. The control group consisted of a parallel large series of pregnant women given conventional ferrous sulphate three times daily. There was no significant difference in the incidence of anaemia, side-effects and complications in the two groups. The once daily regime was as good as, but no better than conventional ferrous sulphate in the prevention of anaemia, although there may be more rapid response in patients with established anaemia.

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Untersuchungen über den Einfluss von Natrium-Oleat auf die Aggregation menschlicher Thrombozyten

A. M. Ehrly, H. Döppenschmidt und E. Böhle

Die Bedeutung der Blutfette für die Entstehung von Thrombozytenaggregaten im Zusammenhang mit Störungen der Hämostase und der Mikrozirkulation ist in den letzten Jahren zunehmend diskutiert worden. Besondere Beachtung verdienen hier die unveresterten Fettsäuren des Plasmas (UFS), die sowohl unter physiologischen als auch unter pathophysiologischen Bedingungen erheblichen Konzentrationsänderungen unterliegen. Über den Einfluss erhöhter Konzentrationen von langkettigen unveresterten Fettsäuren, speziell von Ölsäure auf die Plättchenaggregation liegen allerdings widersprüchliche Angaben vor [1 5 15 16]. Nach eigenen Beobachtungen bewirken sowohl der Zusatz von Ölsäure oder Natrium-Oleat zu Blut *in vitro* als auch eine Erhöhung der Konzentration der unveresterten Fettsäuren *in vivo* eine Desaggregation von Erythrozytenaggregaten [9 10 13]. In der vorliegenden Arbeit wurde untersucht, ob der Zusatz von Natrium-Oleat zu plättchenreichem Plasma eine Aggregation der Thrombozyten *in vitro* bewirkt, und ob verschiedene Konzentrationen von Natrium-Oleat eine ADP induzierte Thrombozytenaggregation verstärken oder abschwächen können.

Material und Methode

Nüchternblut gesunder Probanden beiderlei Geschlechts zwischen 17 und 41 Jahren wurde durch Punktion der Vena cubitalis gewonnen, wobei die ersten ml verworfen wurden.

Herrn Prof. Dr. W. Siede zum 60. Geburtstag

Das Blut wurde in silikonierten Gläschen aufgefangen, in denen so viel Natrium-Zitrat-Lösung vorlag, dass eine Endkonzentration von 3,8% im Blut erreicht wurde. Plättchenreiches Plasma wurde durch 20 min dauerndes Zentrifugieren des Blutes bei 500 g, plättchenfreies Plasma durch 20minütiges Zentrifugieren des plättchenreichen Plasmas bei 2000 g erhalten. Die Extinktionsmessungen wurden mit einem Eppendorf-Fotometer mit dem Filter 578 durchgeführt. In Abänderung der von Born [4] beschriebenen Methode wurde die Durchmischung des plättchenreichen Plasmas mit Natrium-Oleat bzw. Natrium-Oleat und ADP durch Kippen der Gläschen und Küvetten ersetzt. In der Originalmethode [4] wird das plättchenreiche Plasma durch Rotation eines Stäbchens gerührt (stirring). Die infolge der verwendeten hohen Umdrehungszahl des Rührstäbchens von 1000 U/min verursachte mechanische Belastung der Thrombozyten ist gross genug, um einen Teil der Plättchen zu zerstören [3]. Die dabei freiwerdenden Substanzen, insbesondere ADP können selbst eine Aggregation der Thrombozyten auslösen. In einer Mitteilung von Gross *et al.* [14] wurde die *Bornsche Originalmethode* ebenfalls so verändert, dass keine mechanische Alteration der Thrombozyten mehr eintritt.

Nach Zusatz der einzelnen Substanzen zum plättchenreichen Plasma (PRP) wurden die Gläschen mit Parafilm verschlossen und zunächst 20mal gekippt. Die Extinktionen wurden dann in einminütigen Abständen gemessen, beginnend jeweils 60 sec nach Substanzzugabe. 15 sec vor jeder folgenden Ablesung wurde die Küvette wiederum 5mal gekippt. So konnte bei ausreichender Durchmischung des Plasmas eine Sedimentation der Thrombozytenaggregate vermieden werden [8]. Parallel zu jedem untersuchten plättchenreichen Plasma wurde ein Kontrollplasma des gleichen Probanden mitgeführt, dem anstelle der Testsubstanzen ein gleiches Volumen physiologischer Kochsalzlösung zugegeben wurde.

Alle Versuche wurden bei Zimmertemperatur (20 bis 22° C) und unmittelbar im Anschluss an die Blutentnahmen vorgenommen. Sämtliche Zusätze zum Plasma erfolgten in Form von 0,1 ml Lösung der entsprechenden Substanzen in physiologischer Kochsalzlösung. Dabei wurden die Konzentrationen dieser Lösungen so gewählt, dass 0,1 ml ad 5 ml Plasma die angegebenen Konzentrationen, bezogen auf Plasma, ergaben. Natrium-Oleat (Riedel-de-Haen AG Seelze) wurde in 0,9% NaCl gelöst. Als aggregationsauslösende Substanz wurde das Trinatriumsalz des Adenosin-5-Diphosphats der Fa. C.F. Boehringer und Söhne, Mannheim, in einer Endkonzentration von 2×10^{-4} M/l verwendet. Die Lösung wurde täglich neu hergestellt. Alle Geräte, die mit Blut oder Plasma in Berührung kamen, wurden nach den üblichen Vorschriften silikonisiert. Die Genauigkeit der Extinktionsmethode ergab bei Wiederholungsmessungen eine maximale Abweichung von nur 2,63 % [8]. Ein Schema der Versuchsabläufe zeigt Abb. 1

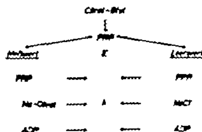


Abb. 1 Schema des Ablaufs der Versuche

Ergebnisse

1 Einfluss von Natrium-Oleat auf die Aggregation von Thrombozyten in plättchenreichem Plasma

Jeweils 8 plättchenreiche Plasmen wurden auf 10 20 40 80 und 160 mg% Natrium-Oleat Konzentration gebracht Parallel zu jedem Messwert wurde ein Kontrollversuch mit physiologischer Kochsalzlösung mitgeführt. Die Beobachtungsdauer betrug 5 Minuten nach Zugabe von Natrium-Oleat bzw physiologischer Kochsalzlösung zu plättchenreichem Plasma. In Vorversuchen [8] hat es sich ergeben, dass eine längere Beobachtungsdauer nicht erforderlich ist.

Die Abb 2 zeigt den Extinktionsverlauf 8 verschiedener plättchenreicher Plasmen nach Natrium-Oleat Zusatz von 20 mg%. Die Extinktionskurven der Kontrollversuche mit physiologischer Kochsalzlösung verliefen deckungsgleich mit den eingezeichneten Kurven. Wie in allen folgenden Abbildungen sind auch hier die Extinktionen der plättchenreichen Plasmen abzüglich der Extinktionswerte der jeweiligen plättchenfreien Plasmen aufgezeichnet worden. Die unterschiedlichen Ausgangshöhen der Kurven sind Ausdruck der in den verschiedenen Plasmen der einzelnen Probanden erhaltenen verschiedenen Anzahl von Blutplättchen. Aus der Abb. 2 ist ersichtlich, dass nach Zugabe von 20 mg% Natrium-Oleat zu plättchenreichem Zitratplasma keine Extintionsveränderung gegenüber den entsprechenden NaCl-Kontrollversuchen erfolgte Analog dazu verliefen auch die Untersuchungen mit 10 40, 80 und 160 mg% Natrium Oleat. In keinem der insgesamt 40 untersuchten plättchenreichen

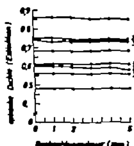
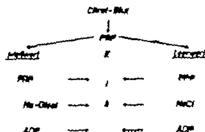


Abb. 2. Das Wirkung von Natrium-Oleat (20 mg%) auf die Aggregation der Thrombozyten in PRP Die Extinktionskurven der NaCl-Kontrollversuche verliefen deckungsgleich mit den eingezeichneten Kurven. Die Ziffern 1–8 bezeichnen die verschiedenen Plasmen.

Das Blut wurde in silikonisierten Gläschen aufgefangen, in denen so viel Natrium-Zitrat-Lösung vorlag, dass eine Endkonzentration von 3,8% im Blut erreicht wurde. Plättchenreiches Plasma wurde durch 20 min dauerndes Zentrifugieren des Blutes bei 300 g, plättchenfreies Plasma durch 20minütiges Zentrifugieren des plättchenreichen Plasmas bei 2000 g erhalten. Die Extinktionsmessungen wurden mit einem Eppendorf-Fotometer mit dem Filter 578 durchgeführt. In Abänderung der von BORN [4] beschriebenen Methode wurde die Durchmischung des plättchenreichen Plasmas mit Natrium-Oleat bzw. Natrium-Oleat und ADP durch Kippen der Gläschen und Küvetten ersetzt. In der Originalmethode [4] wird das plättchenreiche Plasma durch Rotation eines Stäbchens gerührt (*stirring*). Die infolge der verwendeten hohen Umdrehungszahl des Rührstäbchens von 1000 U/min verursachte mechanische Belastung der Thrombozyten ist gross genug, um einen Teil der Plättchen zu zerstören [3]. Die dabei freiwerdenden Substanzen, insbesondere ADP können selbst eine Aggregation der Thrombozyten auslösen. In einer Mitteilung von GROSS *et al.* [14] wurde die Bornsche Originalmethode ebenfalls so verändert, dass keine mechanische Alteration der Thrombozyten mehr eintritt.

Nach Zusatz der einzelnen Substanzen zum plättchenreichen Plasma (PRP) wurden die Gläschen mit Parafilm verschlossen und zunächst 20mal gekippt. Die Extinktionen wurden dann in einminütigen Abständen gemessen, beginnend jeweils 60 sec nach Substanzzugabe. 15 sec vor jeder folgenden Ablesung wurde die Küvette wiederum 5mal gekippt. So konnte bei ausreichender Durchmischung des Plasmas eine Sedimentation der Thrombozytenaggregate vermieden werden [8]. Parallel zu jedem untersuchten plättchenreichen Plasma wurde ein Kontrollplasma des gleichen Probanden mitgeführt, dem anstelle der Testsubstanzen ein gleiches Volumen physiologischer Kochsalzlösung zugegeben wurde.

Alle Versuche wurden bei Zimmertemperatur (20 bis 22° C) und unmittelbar im Anschluss an die Blutentnahmen vorgenommen. Sämtliche Zusätze zum Plasma erfolgten in Form von 0,1 ml Lösung der entsprechenden Substanzen in physiologischer Kochsalzlösung. Dabei wurden die Konzentrationen dieser Lösungen so gewählt, dass 0,1 ml ad 5 ml Plasma die angegebenen Konzentrationen, bezogen auf Plasma, ergaben. Natrium-Oleat (Riedel-de-Haen AG, Seelze) wurde in 0,9% NaCl gelöst. Als aggregationsauslösende Substanz wurde das Trinatriumsalz des Adenosin-5-Diphosphats der Fa. C.F. Boehringer und Söhne, Mannheim, in einer Endkonzentration von 2 x 10-4 M/l verwendet. Die Lösung wurde täglich neu hergestellt. Alle Geräte, die mit Blut oder Plasma in Berührung kamen, wurden nach den üblichen Vorschriften silikonisiert. Die Genauigkeit der Extinktionsmethode ergab bei Wiederholungsmessungen eine maximale Abweichung von nur 2,63% [8]. Ein Schema der Versuchsabläufe zeigt Abb. 1



5 min nach ADP Zusatz ist im allgemeinen das Maximum der Thrombozytenaggregation erreicht (Abb. 3) So wie am Beispiel dieses Einzelversuches mit einer Natrium-Oleat Konzentration von 20 mg% gegenüber der Kontrolle mit NaCl-Zusatz zu plättchenreichem Plasma dargestellt (Abb 3) wurden für jeden Einzelversuch entsprechende Kurven aufgezeichnet, so dass für jede Natrium-Oleat Konzentration jeweils 8 Kurvenpaare erhalten wurden. Aus diesen 8 Kurvenpaaren wurden Mittelwerte errechnet und graphisch auf getragen (Abb. 4 und 5)

Die Abb 4 zeigt die arithmetischen Mittelwerte der Extinktionen 8 verschiedener plättchenreicher Plasmen nach ADP Zugabe.

Bei Zusatz von Natrium-Oleat in einer Konzentration von 10 mg lagen die Extinktionswerte nach ADP Zugabe geringgradig höher als bei den *Kontrollversuchen, was einer Verringerung der Thrombozytenaggregation entspricht.*

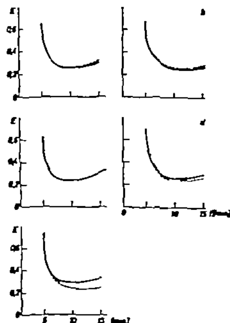


Abb. 4 Kurven der arithmetischen Mittel der Extinktionswerte (E) je 8 verschiedener PRP nach ADP-Zugabe (2×10^{-4} м/l). —— Plättchenreiche Plasmen mit Natrium-Oleat in einer Konzentration von 10 mg% ( ) 20 mg% (b) 40 mg% (c), 80 mg% (d), 160 mg% (e), —— Vergleichsproben der gleichen Plasmen, die NaCl anstelle von Natrium-Oleat enthalten. Natrium-Oleat bzw NaCl wurden bei t = 0 min eingpipettiert, ADP bei t = 5 min.

Plasmen wurde durch Natrium-Oleat Zusatz eine Extinktionsänderung gefunden. Auch mikroskopisch waren nach Natrium-Oleat Zusatz bis 80 mg% keine Thrombozytenaggregate nachzuweisen, lediglich bei einer Plasmakonzentration von 160 mg% Natrium-Oleat waren vereinzelt Thrombozytenaggregate zu finden.

2. Einfluss verschiedener Natrium-Oleat Konzentrationen auf die ADP induzierte Thrombozytenaggregation

Jeweils 8 plättchenreiche Plasmen wurden auf 10 20 40 80 und 160 mg% Natrium-Oleat Konzentration gebracht. 5 min nach dem Zusatz von Natrium-Oleat zu den plättchenreichen Plasmen wurde ADP Lösung zupipettiert. Wie sich aus Vorversuchen ergab, ist es unerheblich, ob zunächst ADP und dann Natrium-Oleat dem plättchenreichen Plasma zugesetzt wird oder ob umgekehrt vorgegangen wird [8]. Die Wirkung von Natrium-Oleat auf eine ADP-induzierte Aggregation von Thrombozyten ist aus dem Vergleich zu den in jedem Fall mitgeführten Kontrollversuchen (Zusatz von physiologischer Kochsalzlösung anstelle von Natrium-Oleat Lösung) ersichtlich. Es sollte so nachgewiesen werden, ob eine ADP-induzierte Thrombozytenaggregation durch Zusatz von Natrium-Oleat in verschiedenen Plasmakonzentrationen verstärkt oder vermindert wird.

Wie Abb 3 zeigt, kommt es innerhalb der ersten beiden Minuten nach Zusatz von ADP zu plättchenreichem Plasma zu dem bekannten Extinktionsabfall (Absinken der optischen Dichte). Gleichzeitig lassen sich schon makroskopisch Thrombozytenaggregate nachweisen.

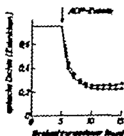


Abb. 3. Änderung der Extinktion eines PRP nach ADP-Zusatz (2×10^{-6} m/l). ●–●–● PRP mit Natrium-Oleat in einer Konzentration von 20 mg%. ○–○–○ PRP mit NaCl anstelle von Natrium-Oleat. Natrium-Oleat bzw. NaCl wurden zur Zeit 0 eingepipettiert, ADP 5 min später

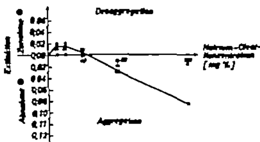


Abb 5. Extinktionsdifferenzen zwischen Mess- (Natrium-Oleat-) und Kontroll- (NaCl-) Plasmen in Abhängigkeit von den geprüften Natrium-Oleat-Konzentrationen. Die schwarzen Quadrate geben die Mittelwerte der Extinktionsdifferenzen an. Ordinate: Unterschiede der optischen Dichte zwischen Mess- und Kontrollplasmen, gemessen in Einheiten der Extinktionsskala des benutzten Gerätes.

tistische Signifikanz sowohl für die desaggregierende Wirkung von Natrium-Oleat in Konzentrationen von 10 und 20 mg% als auch für die aggregierende Eigenschaft von Natrium-Oleat Konzentrationen über 40 mg% ist aus der Abb 5 deutlich ersichtlich (Zeichentext) eine weitergehende statistische Prüfung hatte sich deshalb erübrigt.

Diskussion

Der Ölsäure, als einer einfach ungesättigten C_{18}-Fettsäure, kommt deswegen besondere Bedeutung zu, weil sie von den im Plasma vorkommenden unveresterten Fettsäuren den höchsten Anteil ausmacht [19] In menschlichem Plasma, das bei einem pH von 7 4 eine grosse Pufferkapazität besitzt, sind die unveresterten Fettsäuren nahezu vollständig dissoziiert, da Fettsäuren als Essigsäure-analoge Carbonsäuren einen pK (wässerige Lösung) von 4 7 bis 5 0 aufweisen. Der Zusatz von Natrium-Oleat zu Blut in Konzentrationen bis 120 mg% verändert den pH des Blutes bzw. Plasmas nicht [12] die Wirkungen auf Erythrozyten [13 12, 10] und Thrombozyten sind demnach dem Fettsäureanion zuzuschreiben.

Aus den vorliegenden Ergebnissen geht hervor dass Zusatz von Natrium-Oleat zu Plasma in Konzentrationen bis 40 mg/100 ml eine partielle Desaggregierung von Plättchenaggregaten bewirkt. Hohe Konzentrationen von Natrium-Oleat über 40 mg% verstärken demgegenüber die ADP induzierte Thrombozytenaggregation. Diese

Befunde stehen teilweise im Widerspruch zu den Ergebnissen anderer Autoren. Hierfür dürften an erster Stelle methodische Unterschiede verantwortlich sein. Von HOAK *et al.* [16] sowie MAHADEVAN *et al.* [18] *wurde nachgewiesen*, dass Natrium-Oleat sowohl auf gewaschene Thrombozyten als auch auf Thrombozyten in plättchenreichem Plasma aggregationsfördernd wirkt. Relativ niedrige Natrium-Oleat-Konzentrationen (bis 60 mg /) aggregierten nach weiteren Feststellungen jedoch lediglich gewaschene Thrombozyten, nicht aber Blutplättchen in Plasma [16]. Die gleichen Verfasser konnten weiterhin nachweisen, dass die aggregierende Wirkung des Natrium-Oleates auf gewaschene Thrombozyten durch vorherigen Zusatz von ausreichend Albumin nahezu aufgehoben werden kann. ARDLIE *et al.* [1] fanden bei niedrigen Oleat-Konzentrationen weder eine aggregierende Wirkung des *Natrium-Oleates in plättchenreichem Plasma* noch eine *Beeinflussung* der ADP-induzierten Plättchenaggregation. Die Anwesenheit von Serumalbumin ist offenbar bei *in vitro*-Untersuchungen über die Plättchenaggregation von entscheidender Bedeutung, da dem Plasma zugesetzte Ölsäure bzw. Natrium-Oleat nicht frei vorliegt, sondern an Albumin gebunden wird [11, 16]. Auch die unveresterten Fettsäuren des Plasmas *in vivo* sind an Albumin gebunden. «freie» Ölsäure (ohne Bindung an Plasmaalbumin) kommt *in vivo* praktisch nicht vor. Die fehlende aggregierende Wirkung von Natrium-Oleat in Konzentrationen unter 40 mg% auf im Plasma suspendierte Thrombozyten dürfte daher Folge einer Symplexbildung zwischen dem Albumin und dem Oleat-Anion sein, so dass es nicht zu einer direkten Beeinflussung von freiem, nicht an Albumin gebundenem Natrium-Oleat auf die Blutplättchen kommt.

Unter diesen Gesichtspunkten sind auch die Ergebnisse von HOAK *et al.* zu erklären, die das Auftreten von Thromben in den Lungen von Versuchstieren nach intravenösen Injektionen von Albuminlösungen mit hohen Natrium-Oleat-Konzentrationen beschrieben [15, 16]. *Möglicherweise bestand bei der grossen Menge von zugesetztem* Natrium-Oleat zur injizierten Albumin-Lösung keine quantitative Bindung der Fettsäureanionen an das Albumin, so dass freies, nicht gebundenes Natrium-Oleat direkt auf die Plättchen einwirken konnte. Ähnliche Vorbehalte wie für die Arbeiten von HOAK *et al.* gelten auch für die Ergebnisse von ZAHIDEH [20] und DAY [6], die nach Natrium-Oleat-Infusionen bei Tieren eine Thrombopenie beobachteten.

Intravital erfolgt der Anstieg der Konzentration der unveresterten Fettsäuren bei Stresszuständen, beim Rauchen und während

des Hungers relativ langsam und auf das gesamte Plasmavolumen verteilt. Bei der therapeutischen Anwendung von Heparin und Heparinoiden kommt es zu einem relativ schnellen Anstieg der unveresterten Fettsäuren im Plasma, wobei die Fettsäuren quantitativ an Albumin gebunden sind. Die durch Heparininjektionen ausgelöste *Erhöhung der Konzentration an unveresterten Fettsäuren übersteigt* jedoch im allgemeinen nicht 40 mg% und liegt demnach in einem Konzentrationsbereich, der nach den vorliegenden Ergebnissen eine leichte desaggregierende Wirkung auf Thrombozytenaggregate aufweist.

Die partiell desaggregierende Wirkung von Natrium-Oleat in *Konzentrationen bis 40 mg% auf ADP* induzierte Thrombozytenaggregate zeigt eine auffallende Parallelität zu der früher beschriebenen desaggregierenden Wirkung von Natrium-Oleat auf Erythrozytenaggregate [13] Während jedoch für Erythrozytenaggregate bei höheren Konzentrationen die desaggregierende Wirkung zunimmt, ist dies bei Thrombozytenaggregaten nicht der Fall (s. Abb 5) Die Ursache der thrombozytendesaggregierenden Wirkung von Natrium-Oleat in geringen Konzentrationen und der aggregationsfördernde Effekt von hohen Konzentrationen von Natrium-Oleat konnten bisher nicht gefunden werden. Bei Erythrozytenaggregaten erfolgt die Desaggregation über eine Abrundung der Zellen (bikonvexe Erythrozyten) Der Nachweis eines entsprechenden oder ähnlichen Mechanismus für die Thrombozytendesaggregation nach Zusatz geringer Konzentrationen von Natrium-Oleat gelang mit den üblichen Methoden (Phasenkonstrastmikroskop) nicht.

Der Hemmung der Aggregation der Thrombozyten kommt im Zusammenhang mit der Prophylaxe und der Therapie thromboembolischer Erkrankungen und möglicherweise auch bestimmter Schockzustände eine besondere Bedeutung zu, da die Zusammenlagerung der Thrombozyten einen maßgeblichen pathogenetischen Faktor dieser Erkrankungen darstellt. Nach Deutsch [7] Böhle *et al.* [2] Zweifler [21] und anderen Autoren ist weiter die Möglichkeit zu diskutieren, dass arteriosklerotischen Frühveränderungen über eine Minderung der Thrombozytenaggregation zu begegnen ist. Die vorliegenden Untersuchungen mit Natrium-Oleat ergaben eine leicht desaggregierende Wirkung auf Thrombozytenaggregate bei physiologisch denkbaren Konzentrationen. Den Salzen anderer unveresterter Fettsäuren (z. B. Palmitinsäure, Stearinsäure) wird eine aggregationsfördernde Wirkung auf Thrombozyten nachgesagt [5 16 17 18] Dabei wurden aller

dings relativ hohe Fettsäurekonzentrationen untersucht. Angesichts der Tatsache, dass Natrium-Oleat ein bivalentes Verhalten in Beziehung auf die Thrombozytenaggregation zeigt, erscheinen entsprechende Untersuchungen mit niedrigen Konzentrationen anderer Fettsäuren angezeigt.

Zusammenfassung

Mit einer Modifikation der Bornschen Methode zur Messung der Thrombozytenaggregation wurde die Wirkung verschiedener *Konzentrationen von Natrium-Oleat sowohl auf* Thrombozyten als auch auf ADP-induzierte Thrombozytenaggregate untersucht. Es ergab sich, dass Natrium-Oleat Konzentrationen bis 80 mg/100 ml Plasma keine Aggregation *der Thrombozyten verursacht. Die Wirkung von Natrium-Oleat auf bereits bestehende* Thrombozytenaggregate ist verschieden. Während niedrige physiologisch denkbare Konzentrationen bei 40 mg% eine leichte Desaggregation von Thrombozytenaggregaten *bewirken, führen Natrium-Oleat-Konzentrationen über 40 mg% zu einer Verstärkung der* bereits vorhandenen Thrombozytenaggregation.

Summary

Aggregation of platelets has been studied *in vitro* by a modification of Born method. It could be shown, that addition of sodium-oleat to platelet rich human plasma does not cause platelet aggregation in concentrations up to 80 mg%. When platelets in plasma had been aggregated by ADP before, the addition of sodium oleate in concentrations up to 40 mg% leads to a partial desaggregation of the platelet aggregates, while higher concentrations enhance the aggregation of platelets.

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The Effect of Adrenergic Blockers on the Action of Adrenaline on the Factor VIII Activity in the Plasma

C. Gardikas, C. Lyberatos, J. Pyrovolakis and A. Papayannis

The observation of Ingram [4] that the intravenous infusion of adrenaline brings about a considerable elevation of the factor VIII activity in the plasma of normal subjects, was confirmed by other workers [3 7]. According to Ingram, isopropylnoradrenaline and noradrenaline have no similar effect. The mechanism of this action of adrenaline remains unexplained.

Ahlquist [1] has first shown that the various actions of the sympathomimetic drugs are mediated through two special receptor groups, the α and β receptors. Adrenaline acts on both types of receptors, noradrenaline on the alpha, whilst isopropylnoradrenaline almost exclusively on the β receptors. Various agents block selectively the action on the α or the β receptors. Thus, tolazoline, phentolamine, benzodioxane, dibenamine and phenoxybenzamine are α-adrenergic blocking agents, whilst dichloroisoprenaline, pronethalol and propranolol are β adrenergic blocking agents.

The present paper describes investigations on the effect of α- or β-*adrenergic blocking* agents on the action of the adrenaline on the factor VIII activity in the plasma.

Material and Methods

Experiment I. Ten normal adult males were used. ( ) Adrenaline was administered i. (160 μg in 10 ml of 5% glucose in 10 min). Before and 5 min after the end of the infusion venous blood was collected. (b) Two days later phentolamine (Regitine ®), as a adrenergic blocker was given by i. infusion (10 mg in 200 ml of 5% glucose in 30 min). On the 20th minute of the phentolamine infusion adrenaline was administered as described above in

10 min through vein of the other arm. Five min after the end of the infusion venous blood was collected.

Experiment II. Ten normal adult males were used. ( ) Adrenaline was administered as described in part ( ) of experiment I Before and 5 min after the end of the infusion venous blood was collected. (b) Two days later and for three consecutive days the subjects received orally propranolol (Inderal®) 60 mg daily divided into 4 six-hourly doses. On the fourth day immediately after the morning propranolol dose adrenaline was injected as described in part ( ) of experiment I Five min after the end of the adrenaline infusion venous blood was collected.

Assay of factor VIII Samples of 9 ml of venous blood taken with siliconized needles and glass syringes were mixed with 1 ml of trisodium citrate ($Na_3C_6H_5 \cdot 2H_2O$ 3.8 g/100 ml) in siliconized glass test tube. After centrifugation for 15 min at 300 rpm the plasma factor VIII concentration was assayed immediately by the method described by BIGGS and MACFARLANE [2]. As reference standard mixed pool of plasma from 10 normal donors stored in 1 ml amounts t —30° C was used.

Results

As the tables I and II show neither Regitine® and α-adrenergic blocker nor propranolol, a β-adrenergic blocker inhibit the effect of adrenaline on the factor VIII activity of plasma.

The present experiments support the view that the action of adrenaline on the factor VIII activity of the plasma is not mediated through the special receptor groups, as special adrenergic blockers do not inhibit this action. Thus, the mechanism of the rise of factor VIII activity in the plasma produced by adrenaline remains obscure.

Table I. The effect of Regitine® on the action of adrenaline on the factor VIII activity

Experiment	Factor VIII activity in the plasma (% of normal)		
	before	after administration of	
	adrenaline	adrenaline	adrenaline + regitine
1	98	185	197
2	102	212	208
3	114	265	253
4	94	206	220
5	108	228	221
6	115	301	307
7	96	183	194
8	101	216	209
9	100	182	202
10	107	227	220

The figures represent the mean value of 3 determinations.

Table II. The effect of propranolol on the action of adrenaline on the factor VIII activity

Experiment	Factor VIII activity in the plasma (% of normal)		
	before adrenaline	after administration of adrenaline	adrenaline + propranolol
1	96	214	220
2	102	176	190
3	112	235	228
4	98	206	210
5	98	197	191
6	102	290	283
7	110	198	211
8	98	265	262
9	108	179	188
10	102	221	214

The figures represent the mean value of 3 determinations.

Discussion

The present experiments show that neither phentolamine, an α-adrenergic blocker nor propranolol a β-adrenergic blocker inhibit the effect of adrenaline on the factor VIII activity. These results are in agreement with the findings of McCLURE *et al.* [6] that α-adrenergic blockade with phentolamine did not prevent elevation of factor VIII after adrenaline infusion. On the other hand INGRAM [5] found that pronethalol a β-adrenergic blocker inhibits the elevation of factor VIII brought about by adrenaline infusion. No plausible explanation for the discrepancy of these findings can be given.

Acknowledgement

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Summary

Phentolamine an α-adrenergic blocker and propranolol, β-adrenergic blocker do not inhibit the effect of adrenaline on the factor VIII activity in the plasma.

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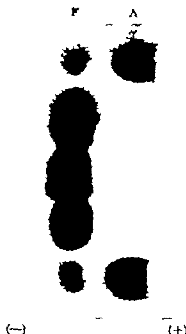


Fig. 2. Agar gel electrophoresis in citrate buffer pH 6.0. At the top and the bottom: haemoglobin of the parents of the propositus. In the center haemoglobin of the propositus and of the other two children, homozygous carriers of *ββ*-thalassaemia. Total absence in these three subjects of haemoglobin A_1 and presence of haemoglobin F only

S. Concetto (II-1, table I, fig. 1) the *father of the propositus* was a healthy subject which showed haematologic picture typical of microcythaemia and a haemoglobin pattern with an elevated level of haemoglobin F (10%) and normal level of haemoglobin A_2 (2.02%). Acid elution test showed quite an heterogeneous intracellular distribution of haemoglobin F

A. Domenica (II-2, table I, fig. 1) *the mother of the propositus* and first cousin of the father was a healthy woman with haematologic microcythaemic picture and haemoglobin pattern with levated haemoglobin F (7.30%) normal haemoglobin A_2 (2.30%), and *heterogeneous intracellular distribution* of haemoglobin F

A. Gregorio (I-1, table I, fig. 1) *paternal grandfather of the propositus*, was healthy microcythaemic subject with the *same* haemoglobin characters of the son (haemoglobin F 9.55% haemoglobin A_2 1.87%) and the same heterogeneous aspect of the cellular distribution of haemoglobin F

Discussion

Both the parents and the paternal *grandfather of the patient* observed presented the haematologic abnormalities typical of microcythaemia (or thalassaemia) with high level of haemoglobin F and heterogeneous erythrocytic distribution of haemoglobin F. These data

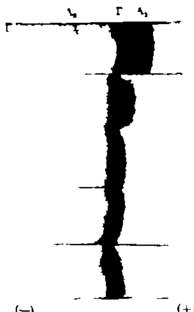


Fig. 3. Cellulose acetate electrophoresis, glycine buffer pH 8.8. From the top to the bottom: the father of the propositus, Cooley disease patient, the three sisters, homozygous carriers of βδ-thalassaemia. Total absence of haemoglobin A_2 and haemoglobin A_1 in the three homozygous subjects.

are typical of F microcythaemia. The three children show a microcythaemic haematologic picture and their haemoglobin is only haemoglobin F. These data suggest that the children are homozygous for F microcythaemia. From the clinical point of view the propositus was the only one to present a slight anaemia with moderate hepatosplenomegaly; the other two sisters are in good health, and have no history of illness.

This is the third family reported in the literature in which cases of homozygosity for F microcythaemia have been found. In the first family [20] the propositus was a 31 year-old woman with slight pallor and marked hepato-splenomegaly; in the second case [22] the condition was discovered accidentally in a 9-year-old boy. Thus, all these observations agree in showing that the homozygous condition for F microcythaemia causes the subjects to be far less severely affected than the homozygous carriers of A_2-microcythaemia.

The same tendency towards a milder clinical picture is also found in the patients with double heterozygosity for A_2- and F microcythaemia.

The cases so far observed include 8 patients with severe Cooley's disease [1 8 16 17] and at least 7 other patients with a much less serious disease (microcytic constitutional anaemia or thalassaemia intermedia) [5 17–19] In another series [12 22] most of the 21 patients were moderately affected

These data show that the F microcythaemia is a microcythaemia variant which interacts with classical A_2 microcythaemia and besides this, they offer the documentation of new variants of genetic basis of the thalassaemia intermedia that is the homozygosity for βδ-microcythaemia with high F and the double heterozygosity for this microcythaemia variant and classical A_2 microcythaemia.

The present patients and the two of the literature had haemoglobin F only Homozygosity for F microcythaemia is thus the third homozygous condition so far described, in which there is a complete absence of haemoglobin A_1 and haemoglobin A_2, the other two being homozygous condition for haemoglobin Lepore and homozygous condition for hereditary persistence of foetal haemoglobin and the second condition in which haemoglobin F only is found (the other being homozygosity for high F gene) This proves that in the three families so far described the βδ-microcythaemia genes completely suppress β- and δ-chain synthesis.

There are, till now only few cases which can supply informative data on the activity of the structural genes β and δ in this variant of microcythaemia The three present and the two preceding homozygotes [10 21 22] and the double heterozygote for B_2 haemoglobin and βδ-microcythaemia [14] show a complete inactivity of locus δ As for locus β in the 5 homozygotes for F microcythaemia and in the 2 double heterozygotes for haemoglobin S and F microcythaemia [4 6] such locus has proved to be completely inactive in 6 cases and partially functioning in 1 case [4] It would appear that in F microcythaemia, likewise in A_2 microcythaemia, locus β is in most instances suppressed and rarely partially functioning as for locus δ on the contrary the observations are not sufficient to prove a constant and complete inactivity A larger number of cases is necessary for the final interpretation of the genic effects of this microcythaemic mutation.

In βδ-microcythaemia with high F locus γ appears affected in a characteristic way there is, in the heterozygote state, an evident hyperactivity which has such a constant uniformity as to appear genetically determined On the contrary the resulting haemoglobin F is irregularly distributed in the erythrocytes – like in the other forms of

microcythaemia – a fact that would suggest a compensatory mechanism. Therefore the compensatory hyperactivity of locus γ is typically evident in the F microcythaemia heterozygote, but not in the A_2-microcythaemia heterozygote. Moreover in the $\beta\delta$-microcythaemia homozygote the hyperactivity of locus γ often reaches a much higher functional level than in the classical β-microcythaemia with high A_2 homozygote thus considerably reducing the severity of clinical manifestations.

This consideration and the equality of the haemoglobin picture of homozygous carriers of F microcythaemia and of hereditary persistence of haemoglobin F brings to the fore the problem of relationships between microcythaemia and hereditary persistence of haemoglobin F. The similarity between the two conditions is highly suggestive. It is however clear that the two conditions cannot be based on the same genetic alteration. While in homozygous F microcythaemia state the degree of haemoglobinization is always below the normal range (so that the haematological picture of microcythaemia is always present) in homozygous state for high haemoglobin F gene the normal haemoglobin level reveals that loci γ are both fully functioning.

Therefore in F-microcythaemia any future hypothesis on the basic genetic mechanism shall have to take into account not only the total or partial suppression of the products of the two adjacent structural genes β and δ but also the partial and characteristic persistence of activity of structural gene γ which is constant and typical in this microcythaemia variant.

Summary

In a family of Sicilian origin the parents, who are first cousin to each other are both carriers of $\beta\delta$-thalassaemia (or microcythaemia) with high haemoglobin F (F-thalassaemia) and their three children are homozygous carriers of F-thalassaemia. Clinically only the propositus shows a slight anaemia and a moderate hepato-splenomegaly. His two sisters are apparently healthy. As the two preceding observations of literature, this family proves that the activity of normal β and δ genes is completely suppressed in F-thalassaemia, and that the homozygous condition produces a much less severe disease than the homozygosity for β-thalassaemia with high haemoglobin A_2 genes.

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Complete Remission in Chronic Lymphocytic Leukemia

Report of a Case

P. Pardo, E. Simó, M. Ribas-Mundó and P. Farreras

Patients with chronic lymphocytic leukemia have usually a long survival either spontaneously or with treatment, but lymphocytic infiltration of the bone marrow and blood lymphocytosis persist in the majority of cases throughout the course of the disease.

We report a patient with chronic lymphocytic leukemia in whom complete clinical and hematological remission, proven by even cytochemical and lymphographic techniques, developed after therapy with cyclophosphamide and prednisone. After the complete remission it was impossible to find any feature which let us suspect the persistence of chronic lymphocytic leukemia. As this is an unusual case not often reported in the literature we think it might be worth to report it.

Case Report

A 63 years old, married, railroad employé, born in Peñalba (Huesca), Spain. Family history was not significant. At the age of 24 he suffered pleuritis of unknown etiology. At the age of 35 ocular surgery for strabismus. Since then he has complaint of frequent upper respiratory infections.

His present illness began at the age of 63 (February 1961) with bronchitis, progressive hemoptoic sputum and acute and severe pain of the left eye followed by amaurosis. A diagnosis of glaucoma was made. At that time an hemogram revealed no anemia, 15,770 leukocytes with 96% lymphocytes and numerous Gumprecht cells.

Physical examination revealed an apparently healthy man with amaurosis of the left eye and generalized lymphadenopathy of medium size in cervical, axillary and inguinal regions.

Laboratory analyses (24.II.61): E.S.R. 11 mm at the first hour; red blood cell count 4,565,000; hemoglobin 13.6 g%; total leukocyte count 15,770 with juvenile forms 1%,

segmented neutrophils 3%, lymphocytes 96% and many Gumprecht cells. Myelogram hypoplastic erytropoiesis of about 8% of the total cellularity 80% of infiltrating mature lymphocytes and some lymphoblasts, scattered myelocytes and metamyelocytes. No megakaryocytes. Total proteins 5.6 g%, albumine 3.08 g%, globuline 1.24 g%. Uric acid 8.55 mg%. Platelet count 230,000 Due to the good general condition of the patient no specific treatment was given.

Three months later (3.VI.61) the leukocyte count rose to 147,070 and an enlarged spleen was detected 4 cm under the left costal margin. Treatment was started with 150 mg cyclophosphamide and 10 mg prednisone per os daily and was given for 3 months. I September 1961 the spleen was not palpable and the leukocyt count had dropped to 14,080. Treatment was reduced to 200 mg cyclophosphamide i.m. weekly and 5 mg prednisone per os daily

During this time he suffered from frequent upper respiratory infections. A basocellular epithelioma infiltrating the left preauricular region and spinocellular epithelioma on the right temporal region also developed.

After year of treatment (8.V.62) with cyclophosphamide and prednisone the peripheral leukocyte count was 11,500 with 57% lymphocytes. The bone marrow aspirat revealed 20% lymphocytes. On physical examination the liver was still palpable 3 cm below the right costal margin and axillary lymph nodes were still felt. The same treatment was prescribed.

I May 1964, three years after beginning of treatment, the patient was in good condition. On physical examination only the liver was palpable 2 cm below the costal margin neither splenomegaly nor lymphadenopathies were detected. Red blood cell count was 5,200,000, Hb 14.7 g% and the leukocyte count was 8,250 with 22% lymphocytes. Trea ment with cyclophosphamide 50 mg and prednisone 5 mg per os every other day was continued. I December 1964 the liver was no longer palpable. Analysis of peripheral blood was normal and treatment was stopped.

I July 1966 physical examination was normal. The erythrocyte count was 5,300,000 with 16.1 g% Hb, the leukocyt count was 7,370 with 36% lymphocytes; only 15% of these lymphocytes presented PAS reaction. The bone marrow aspiration was normal with 5% lymphocytes.

I July 1967 the patient was asymptomatic and physical examination was entirely normal. The hematological data remained within the normal limits. Less than 10% of the peripheral lymphocytes presented PAS reaction. The bone marrow aspiration revealed normal erythro- and leukopoiesis, presence of megakaryocytes and 12% of lymphocytes. The lymphography in July 1967 failed to reveal enlarged retroperitoneal lymph nodes.

I March 1968 the patient is still in good clinical condition. Neither hepato-splenomegaly nor lymphadenopathies are palpable. Nevertheless, peripheral blood leukocyte count has risen to 15,560 with 52% lymphocytes. Bone marrow aspiration reveals 30% lymphocytic infiltration. Treatment with cyclophosphamide 50 mg and prednisone 5 mg every other day is reinstaured.

Discussion

Chronic lymphocytic leukemia has always been considered the less malignant of all types of leukemia. Patients with long survival are common. Our case, however has remained in complete remission for at least 3 years with no clinical or laboratory features of chronic lymphocytic leukemia.

In the available literature we have found very few cases were the complete remission of chronic lymphocytic leukemia had been satis-

factorily proven. REICH [1] published in 1959 a case of complete remission after radiotherapy of the spleen (1 700 r) and blood transfusions. The patient persisted on complete remission for 5 years. DURANT and FINKBEINER [2] published in 1964 a revision of 250 cases of chronic lymphocytic leukemia, lymphosarcoma and leuko-lymphosarcoma. These authors comment the difficulties encountered in some cases to differenciate chronic lymphocytic leukemia and lymphosarcoma. From the 250 cases only one achieved complete remission during 3 years after treatment with prednisone and TEM. In 1955 a case of remission in a female treated with radiotherapy was published by SCHOTT [3]. However there is no mention of the myelogram during the remission phase. Recently TEN HAN et al. [4] have described 6 cases of complete remission among 202 patients with chronic lymphocytic leukemia and leukolymphosarcoma. The lymphocytic infiltration in bone marrow of these cases during remission ranged however between 15 and 20 % which is over the upper limit for normal adults.

In our case the diagnosis of chronic lymphocytic leukemia seems inequivocal for he had generalized adenopathy, liver and spleen enlargement, white cell count of 147 000 with 96 lymphocytes and many Gumprecht cells, bone marrow infiltration with mature lymphocytes and some lymphoblasts. The leukocytosis of 13 560 with 52 lymphocytes and the 80 lymphocytic bone marrow infiltration after 3 years of clinical and hematological remission confirms the diagnosis of chronic lymphocytic leukemia.

We consider a complete remission to be present when there are no clinical or laboratory signs which could detect the disease. These criteria are consistent with the published criteria of BISEL [5] for complete remission of acute leukemia. We suggest two further criteria to accept the complete remission of chronic lymphocytic leukemia: (1) The PAS reaction of the peripheral lymphocytes should be positive in less than 20 as is in normal subjects; (2) the lymphography should be normal.

In our patient complete remission during 3 years can be proved by the following data: (1) No clinical symptoms of disease; (2) no palpable adenomegaly; no hepatoesplenomegaly; (3) red blood cell count 5 300 000, Hb 102, white blood cell count 7 370 with 36 lymphocytes; (4) the lymphocyte content of the bone marrow is less than 15; (5) less than 20 of the peripheral lymphocytes show a PAS reaction; (6) the lymphography is normal.

The maintenance dose of cyclophosphamide and prednisone even when the patient was in good condition has probably played a role in the evolution of this patient, but as Tin Han *et al* [4] comment, factors other than treatment might mediate in such cases. In accordance with these authors it is also possible that the frequency of complete remission in chronic lymphocytic leukemia might be increased significantly if patients were treated earlier and with a maintenance dosage of proven effective drugs in a long term therapy

At the Department of Hematology of the University of Barcelona we have only seen this single case of complete remission among 68 cases of chronic lymphocytic leukemia in a period of 10 years.

Summary

The authors report case of chronic lymphocytic leukemia which attained clinical and hematological remission during 3 years after treatment for 3 years with cyclophosphamide and prednisone. A review of the literature has revealed very few cases of complete remission in chronic lymphocytic leukemia. The authors suggest two further criteria of complete remission in chronic lymphocytic leukemia the PAS peripheral lymphocytes should not exceed 20% and the lymphography should be normal.

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Acute Erythromyelosis after Benzene Poisoning

C. Rozman, S. Woessner and J. Saez-Serrania

In 1965 we published 20 cases of aplastic anemia induced by chronic benzene poisoning [1]. Malignant neoplastic disorders of the bone marrow, acute leukemia or less often chronic leukemia, have been found in patients with prolonged exposure to benzene containing environments [2, 3]. There are more than 100 of such cases referred to in the literature [3]. Rare cases of erythromyelosis (erythremia in its pure form or as erythroleukemia) have been found associated with long contacts with benzene, the latter form being more common than the former [3–13]. The rarity of this cases has prompted us to report a patient with acute erythromyelosis after chronic benzene poisoning.

Case Report

A 58-year-old varnisher was seen in January 1968, with a previous history of working during 30 years in a closed environment rich in benzene.

In November 1967 after a short 'flu' with low fever, the patient noticed onset of dyspnea after effort, lassitude and paleness. A blood count performed in early January showed normochromic anemia with 2,500,000 RBC/mm³. On examination hepatosplenomegaly was found. Chest roentgenogram was normal. In two separated occasions 500 ml of whole blood were given, without improvement in the patient's condition.

A hematological study performed on the 15th of February showed the following data: RBC 1,480,000/mm³, hemoglobin 25 ..., Hbg 27 pg, Reticulocytes 1,300/mm³, Platelets 11,000/mm³, Leukocytes 11,000/mm³: myeloblasts 1%, promyelocytes 3%, neutrophilic myelocytes 2%, metamyelocytes 7%, young neutrophils 4%, segmented neutrophils 41%, lymphocytes 21%, monocytes 1 ... 3 erythroblasts per 100. Very marked anisopoikilocytosis, some erythrocytes with crenated edges.

Bone marrow: Proerythroblasts 1.4%, basophil erythroblasts 40.0%, polychromatophil erythroblasts 19.0%, ortochromatic erythroblasts 17.2%, myeloblasts 0.8%, promyelocytes 5.8%, metamyelocytes 1.2%, reticulum cells 4.6%. Marked morphological changes of the erythroblasts were found mainly in the nuclei. Giant polyploid forms, diploerythroblasts, atypical mitoses and in some cells cytoplasmatic vacuoles were seen (fig. 1–3). There are

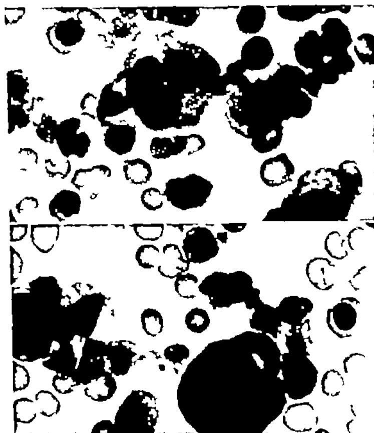


Fig 1 Giant and small polyploid erythroblasts with 4 and 5 nuclei.

no atypism in the white cell series. The number of megakaryocytes was scarce. Most erythroblasts showed an intense diffuse PAS-positivity of the cytoplasm. Some erythroblasts had PAS-positive granules. Perls stain showed 78% sideroblasts without ring forms.

The course of the illness was growing worse, without response to the therapy. The patient died on the 24 of February of massive intestinal hemorrhagy.

Comment

Clinically and experimentally it has been shown that benzene exerts its toxicity upon the erythroblastic mitoses resulting in giant erythroblasts, polyploid mitoses and arrested mitoses in metaphase [11 12 14]. The finding of diploerythroblasts in marrow medular aplasia identifies benzene as the most probable causal agent [15].



Fig 2. Group of young erythroblasts with vacuoles in the cytoplasm.

Fig 3. Three 'diploerythroblasts' with nuclei arranged like coffee beans.

The above named anomalies and the history of prolonged exposure to benzene have lead us to consider this substance as the causative agent. On the other hand the other clinical and hematological findings are similar to the non benzene produced erythremias. The ferrokinetic and erithrokinetic findings [1] and the PAS-positivity of the erythroblasts, as shown in our case are similar in both types of erythremia. There are a number of blood diseases, like thalassemia, iron-deficiency anemia and hemolytic disease of the newborn which show some PAS-positivity in the erythroblasts, but this is more intense in the

acute erythremia (DI GUGLIELMO's disease) and offers an important clue to its diagnosis [16]

Summary

A case of acute erythromyelosis is reported after 30 years exposure to benzene. Nuclear abnormalities were found in the erythroblasts, which showed marked PAS-positivity in their cytoplasm, like in other types of erythremia.

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Erratum

In der Arbeit SCHAFFALITZKY SCHNIDER „Zur Natur der stark basophilen Zellen in der Ductus thoracicus-Lymphe" (Vol. 39 Nr. 5, 1968) wurden leider die beiden Abbildungen vertauscht.

Therapie der Leukosen und malignen Lymphome

In Wien findet vom 24.–26. März 1969 eine internationale Arbeitstagung über „Chemo- und Immunotherapie der Leukosen und malignen Lymphome" statt. Leitung: Prof. Dr. H. FLEISCHHACKER. Auskünfte: Doz. Dr. A. STACHER, Ludwig Boltzmann-Institut für Leukämieforschung und Hämatologie, *A-1140 Wien*, Heinrich Collin-Strasse 30.

International Society of Blood Transfusion

At the meeting of the ISBT Executive Committee which took place in Moscow from the 26th February to the 1st March, it was decided that the Society could offer a few travel grants to help some young scientists to go to the XIIth International Congress of Blood Transfusion, Moscow, August 17–23, 1969. The total amount of money available for travel grants is $ 3000 which will be divided among a few candidates up to the age of 35 years, actively engaged in research, to assist them in paying for their travel expenses.

Applicants shall apply to Dr E. FREIESLEBEN, Blood Bank, Rigshospitalet, 9 Blegdamsvej, *2100 Copenhagen* (Denmark), furnishing the Committee with 4 copies, abstracts or reprints of the paper to be submitted, a complete curriculum vitae (including the age of the applicant) and a letter of recommendation from the applicant's chief or head of the research programme, as well as some information about the applicant's possibility of obtaining financial support from other sources. The applications should be sent before the 1st of February 1969.

Acta haemat. 40: 241–251 (1968)

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Measurements of Protein Synthesis by Lymphocytes *in vitro* in Diseases of the Lymphoid Tissues

J. W. M. Lawton and L. J. Forbes

In earlier studies [8, 9] measurements showed reduced globulin synthesis by lymphocytes from peripheral blood in a variety of disease states. Of particular interest were low values in a few cases of chronic lymphatic leukaemia and macroglobulinaemia, suggesting that the reduced rate of immunoglobulin synthesis commonly found in these conditions [17] may be associated with measurable metabolic deficiency in the circulating lymphocytes.

Studies were therefore undertaken to extend these preliminary observations. The activity of lymphocytes was measured in medium containing autologous plasma and in plasma-free medium, to investigate the possibility that circulating substances may inhibit protein synthesis by lymphocytes in disease states. Parallel measurements were made of the effect of phytohaemagglutinin on protein synthesis. Lymphocytes were studied from normal subjects, and from patients with chronic lymphatic leukaemia, lymphoma (unclassified) and lymphosarcoma, macroglobulinaemia, multiple myeloma and infectious mononucleosis.

Materials and Methods

Normal subjects were healthy laboratory workers, medical practitioners, students, and blood donors. Diagnosis of chronic lymphatic leukaemia (CLL) was made on blood count of more than 9,000 lymphocytes/mm³ and the finding of non-follicular lymphocytic infiltration of the bone marrow [3]. Multiple myeloma was diagnosed by γ- or β-2A band of paraprotein on serum electrophoresis which was not precipitated from the serum by rivanol [15] associated with an infiltration of the bone marrow by plasma cells. I macroglobulinaemia the paraprotein was precipitated from the serum by rivanol, and was associated with

infiltration of the bone-marrow by abnormal cells of the lymphocyte series. The category 'lymphoma (unclassified)' was made up of a small group of patients with lymphocytic infiltration of the bone marrow but without diagnostic changes in the lymph node blood or bone marrow. Diagnosis of lymphosarcoma was made from histological sections. Diagnosis of infectious mononucleosis was made in the presence of characteristic symptoms and signs, and the presence of atypical lymphocytes in the blood [6]. Heterophile agglutinins [12] were present in 6 cases, absent in 3 and not tested for in one case. The heterophile antibodies were not absorbed by guinea-pig kidney suspension [3] in 5 cases and were absorbed in one case.

Culture of lymphocytes. Venous blood, 40–50 ml where possible was collected into heparin (25 IU/ml) and dextran 0.5%. After the erythrocytes had sedimented for 1 h at 37°C, the supernatant plasma was taken and centrifuged at 800 g for 15 min. The sedimented leucocytes were resuspended in half the volume of plasma and incubated at 37°C for 1 h in a glass column packed with cotton wool. The lymphocytes were eluted from the column with the other half of autologous plasma, washed twice with BSS and suspended in Eagle medium supplemented with non-essential amino acids [1]. From each blood sample 12 × 1.0 ml cultures were prepared in 4 groups, so that each determination was made in triplicate. The composition of the cultures is shown in table I. A small volume of the lymphocyte suspension was dispensed separately for the determination of the lymphocyte count (Coulter Counter Model D) and for making a smear for a differential leucocyte count. Cultures were incubated at 37° for 24 h then frozen.

Measurement of protein synthesis. Cultures were frozen and thawed three times and centrifuged at 10 000 g for 15 min to remove cell debris. T each plasma-free supernatant was added 0.25 ml of standard human pooled serum. The protein was precipitated in 5% trichloracetic acid, washed three times, dissolved in NH_4OH and plated at infinite thickness on 2 cm planchettes for counting in an end-window gas-flow Geiger-Müller counter.

Total protein concentration of plasma was estimated by the method of WOLFSON *et al.* [20].

Calculation of results. Net counts per minute (CPM) were obtained by subtracting the CPM of protein in cultures containing puromycin, 60 µg/ml. These samples gave counts very little above background for the counter (15–20 CPM). Mean net CPM of triplicates was converted to 'mean net CPM/10^6 cells.

The following 4 correction factors were applied

1 Correction for the efficiency of the counter

Table I Composition of standard cultures

Group	Cell suspension ml	Autologous plasma ml	PHA ml	^{14}C-leucine solution ml	Final ^{14}C-leucine concentration µCi/ml
Plasma free	0.5	0	0	0.5	0.5
Plasma free + PHA	0.5	0	0.06	0.5	0.5
25% plasma	0.5	0.25	0	0.25	0.25
25% plasma + PHA	0.5	0.25	0.06	0.25	0.25

L-Leucine ^{14}C(U) specific activity 150 mCi/m-mole, Radiochemical Centre Amersham (England)

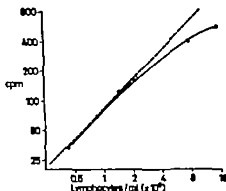


Fig 1 Effect of increasing population density in 25% plasma medium. Simultaneous triplicate 1 ml cultures of lymphocytes from the same subject were set up with increasing numbers of lymphocytes. Protein synthesis was measured after incubation for 24 h (cpm counts per minute).

2. Correction for the ^{14}C-leucine concentration to standard ^{14}C concentration of 0.5 µCi/ml. It was shown that the CPM in newly synthesized protein were directly proportional to the concentration of radioactive substrate.

3. Correction for the amount of carrier protein to correspond to the amount (15.5 mg) in 0.25 ml of standard pooled serum, by reference to standard curve.

4. Correction for population density in plasma-containing cultures to give the theoretical incorporation in culture containing 1×10^6 cells/ml, using the graph shown in figure 1 (No such correction was necessary for plasma-free cultures where the incorporation was directly proportional to the number of lymphocytes per ml up to density of 16×10^6 lymphocytes per ml).

Results

1 Protein synthesis in plasma free medium

Mean total synthesis. The basal (unstimulated) synthesis by normal lymphocytes in plasma-free medium was measured in 28 healthy adults. The mean incorporation was not significantly different from the normal in all clinical groups CLL, lymphoma, lymphosarcoma, macroglobulinaemia, multiple myeloma and infectious mononucleosis (table II)

PHA stimulation of protein synthesis by normal lymphocytes. The stimulation by PHA is expressed as a ratio incorporation with PHA/incorporation without PHA. The effect of PHA in increasing protein synthesis by normal lymphocytes in plasma-free medium was highly significant ($P < 0.001$) The values for PHA stimulation under these culture conditions ranged from 0 7 to 3.5 with a mean of 1.87 (table II)

ing autologous plasma. Also, in order to use radioactive substrate as economically as possible, the incorporation of ^{14}C-leucine into all of the soluble proteins was measured, rather than into the portion which was precipitated by sodium sulphate solution. The fraction of the radioactive proteins in the globulin fraction is quite variable in normal subjects and is usually substantially less than half of the total. The results in the present study are therefore not directly comparable with the previous measurements.

These studies have provided further evidence that circulating lymphocytes may have a reduced capacity to synthesize protein in diseases involving lymphoid tissues.

Measurements of protein synthesis in plasma-free medium alone showed low activity by lymphocytes from all of the disease categories studied except multiple myeloma. However the mean total synthesis in any disease group was not significantly lower than the control mean, nor was the difference significant when the data from all categories was pooled.

The addition of 25 % of autologous plasma to the medium significantly enhanced protein synthesis by normal lymphocytes. This effect is probably due to better nutrition, as unidentified substances in plasma are necessary for optimal growth of other mammalian cells *in vitro* [7] AMBROSE [1] found hydrocortisone to be the factor in serum which was necessary for maximal antibody synthesis by rabbit lymph-node fragments *in vitro* but hydrocortisone has been shown to inhibit protein synthesis by human peripheral lymphocytes [16]

No evidence was found of inhibitory substances in the plasma of subjects with CLL, lymphoma and lymphosarcoma. The mean values for plasma stimulation of protein synthesis by lymphocytes in these conditions were actually higher than the normal mean, although they did not reach the level of statistical significance. These findings are of interest in view of the suggestion of VON HALLAUER [19] that CLL serum contains substances which stimulate cell growth

Although the mode of action of PHA and the significance of the response to it are not yet known, it is a useful substance to bring out differences in lymphocytes from different sources. When the adjuvant effects of plasma and PHA were both utilized it was possible to show significant deficiencies in the capacity of lymphocytes to synthesize protein. Significantly low activity was seen in lymphocytes from subjects with lymphoma, lymphosarcoma, macroglobulinaemia and infectious mononucleosis. Lymphocytes from patients with CLL showed

the same trend and when only the untreated cases were considered the mean incorporation was significantly lower than the normal.

The low total protein synthesis by the circulating lymphocytes in lymphoma, lymphosarcoma and macroglobulinaemia seems to be related to the poor immunological function reported in these so-called lymphoproliferative disorders [3 4 10 17]

Significantly low values for PHA stimulation in medium containing plasma were found in 2 cases of lymphosarcoma. In chronic lymphatic leukaemia one estimation was above the normal range and 3 were below The degree of stimulation by PHA was found to be significantly low in infectious mononucleosis, omitting one case in which the diagnosis was doubtful The low stimulation in infectious mononucleosis agrees well with the lack of increase of RNA synthesis in response to PHA [13] RUBIN also noted that unstimulated lymphocytes in infectious mononucleosis synthesized RNA at 10 times the rate of normal blood lymphocytes under the same conditions. This finding is not compatible with the results in our series where incorporation of C-leucine into protein was not elevated. The reason for this discrepancy is not readily apparent. It may be due to a difference in the stage of the disease between the two series more than 60% of the lymphocytes in RUBIN's cases were morphologically atypical whereas a much lower proportion of such cells was found in the blood of the subjects of this study

The cause and significance of these abnormalities of protein synthesis are not understood. Further qualitative characterization of the deficiencies is necessary It is also necessary to consider the population dynamics of the disorders, for the lymphocytes are a heterogeneous population, at least with respect to immunoglobulin synthesis [18] and each immunologically competent cell normally synthesizes antibodies of only one or two specificities [11 2] The changes in the total protein synthesis may reflect changes in the proportions of various types of cell, o changes affecting each cell of the whole population. The derangement could conceivably be initiated at the level of formation of stem cells, in the thymus, or in the peripheral lymphoid tissues. It seems likely that the disturbance in infectious mononucleosis is self limited, occurring in peripheral lymphoid tissues The disturbance may be at a similar level in lymphosarcoma, and it has been argued that the abnormalities of immunoglobulin synthesis in CLL may also be explained by a failure of terminal immunologic determination at this level [14]

Acknowledgements

We are grateful to Miss Peggy Wood for skilled technical assistance. This research was supported by a grant from the National Health and Medical Research Council of Australia.

Summary

Measurements were made of protein synthesis *in vitro* by lymphocytes from normal subjects and from patients with chronic lymphatic leukaemia, lymphoma (unclassified) and lymphosarcoma, macroglobulinaemia, multiple myeloma and infectious mononucleosis. Protein synthesis was significantly stimulated by both plasma and phytohaemagglutinin in all groups. When the lymphocytes were cultured in medium containing 25 % autologous plasma and PHA, significant differences between the normal and disease groups were observed. The degree of stimulation by phytohaemagglutinin in medium containing 25 % plasma was significantly low in the lymphosarcoma and infectious mononucleosis groups. In interpreting these findings it is necessary to take into account the functional heterogeneity of the circulating lymphocytes.

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Über den Abbau von Zellen im Knochenmark des Menschen und der Ratte

D. Graf Keyserlingk

Der Abbau von Erythrozyten und Erythroblasten in den phagozytierenden Retikulumzellen des Knochenmarks ist bereits sorgfältig untersucht worden [2, 3, 4, 5]. Auch Kerne und Fortsätze von Megakaryozyten wurden in diesen Zellen gefunden [36, 38]. Es fehlen jedoch genaue Angaben darüber, welche Zellen ausserdem noch in den phagozytierenden Retikulumzellen abgebaut werden und wie dieser Vorgang morphologisch erfolgt. Bei der elektronenmikroskopischen Untersuchung dieser Frage zeigte sich darüber hinaus, dass der intrazelluläre Abbau von Zellen im Knochenmark bei Mensch und Ratte zu morphologisch verschiedenen Abbauprodukten führt.

Material und Methode

Menschliches Knochenmark wurde durch Sternalpunktion gewonnen. Ein Teil der Patienten litt an hämatologischen Erkrankungen, während bei einer zweiten Gruppe zunächst der Verdacht auf eine solche Erkrankung bestand, der dann licht- und elektronenmikroskopisch nicht bestätigt wurde. Das Knochenmark der Ratte wurde dem Femur von 6 Tieren eines Wistar-Inzuchtstammes entnommen. Das Gewebe wurde sofort in einer 1% OsO_4-Lösung in Michaelis-Puffer mit Saccharosezusatz bei pH 7,4 und 4°C für 1½ Std. fixiert. Stufenweise Entwässerung in Aceton und Einbettung in Micropal. Zum Nachweis der sauren Phosphatase erfolgte die Fixierung in 2,5% Glutaraldehyd in 0,1 m Cacodylat-Puffer pH 7,4. Weitere Behandlung nach der Vorschrift von Smith und Farquhar [30]. Das Inkubationsmedium enthielt 0,01 m Na-β-Glycerophosphat, 0,004 m $Pb(NO_3)_2$ in 0,05 m Acetatpuffer bei pH 4,9. Die Inkubationszeit betrug 1 Std. bei 37°C. Anschließend Nachfixierung in 1% OsO_4-Lösung in Michaelis-Puffer. Entwässerung und Einbettung wie oben. Die Schnitte wurden mit einem LKB-Ultrotom hergestellt und mit Uranylacetat und Bleicitrat nachkontrastiert. Aufnahmen mit einem Siemens Elmiskop I.

Befunde

Bei der elektronenmikroskopischen Beurteilung von Phagozytosevorgängen muss die geringe Dicke der Schnitte berücksichtigt werden, um Fehldeutungen zu vermeiden. Die Ausläufer der phagozytierenden Retikulumzellen des Knochenmarks reichen oft weit zwischen die benachbarten Zellen. Um eine extrazellulär gelegene Zelle nicht fälschlicherweise als Phagosom anzusehen muss deshalb eine eingeschlossene Zelle nicht nur vollständig von einer Membran der phagozytierenden Zelle umgeben sein, sondern auch Veränderungen in ihrer Feinstruktur die für einen Abbau sprechen, erkennen lassen. Nur bei der Enzymreaktion unterscheidet sich diese Einschlussmembran schon durch ihren Niederschlag von der äusseren Zellmembran. In den phagozytierenden Retikulumzellen des Menschen und der Ratte konnten wir Zellen der roten Reihe, isolierte Normoblastenkerne neutrophile Granulozyten (Abb. 1a) Lymphozyten, Plasmazellen (Abb 1b) und Thrombozyten nachweisen. Da der Abbau hämoglobinhaltiger Zellen bereits mehrfach untersucht wurde [31] braucht hier nicht näher darauf eingegangen zu werden. Das markanteste Zeichen dieses Abbaus ist das Auftreten von Einschlusskörpern mit Ferritin-Granula [24] (Abb. 2) Ferritinhaltige Einschlüsse sind auch in unserem Material häufig zu finden.

Alle Einschlusskörper enthalten das Reaktionsprodukt (Bleiphosphat) der sauren Phosphatase. Wenn eine phagozytierte Zelle noch keine deutlich ausgeprägten Zeichen des Abbaus aufweist, so liegt der Bleiphosphat Niederschlag der umgebenden Membran innen an (Abb 2) Die äussere Zellmembran der phagozytierenden Retikulumzelle ist stets frei von Niederschlägen. Wenn deutliche Abbauerscheinungen in der eingeschlossenen Zelle erkenntlich sind, liegt das Reaktionsprodukt der sauren Phosphatase auch innerhalb des Phagosoms. Kleine Einschlüsse können vollständig von dem Niederschlag ausgefüllt sein.

Die Einschlussmembran kann der phagozytierten Zelle dicht anliegen (Abb 1b) oder einen mehr oder weniger breiten Spaltraum zum Phagosom freilassen (Abb 1a, 3) Dieser Spaltraum erscheint weitgehend elektronenoptisch leer (Abb 3) oder mit einem amorphen Material geringer Elektronendichte ausgefüllt.

Im Kern der phagozytierten Zelle treten Veränderungen auf, bevor andere Abbauerscheinungen deutlich werden. Das Nukleoplasma verliert sein typisches Aussehen und wird pyknotisch (Abb 1a)

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Investigations on the Fibrinolytic System in Liver Cirrhosis[1]

G. Tytgat[2], D. Collen[3], R. De Vreker and M. Verstraete

For nearly half a century various observers have reported increased fibrinolytic activity in the blood of patients with liver cirrhosis. However the fibrinolytic activity was frequently measured by the autodigestion time of clots of total or diluted blood or plasma of the patient. This decreased lysis time, often due to low amounts of lysable fibrin substrate, should be interpreted as 'pseudofibrinolytic activity' as long as the lytic activity cannot be measured on a standard substrate not variable from patient to patient.

Nevertheless in some well documented cases of cirrhosis, true increased plasma fibrinolytic activity has been demonstrated. This enhanced blood proteolytic activity may be a cause of spontaneous bleeding or may encourage bleeding from esophageal varices or surgical wounds [5, 8, 11]. Particularly patients subjected to portocaval shunt operations have an increased tendency to operative and postoperative bleeding [9].

According to Fletcher *et al.* [7] the increased fibrinolytic activity in the cirrhotic patients may be due to a failure of hepatic clearance mechanisms for plasminogen activator and not to deficient plasma fibrinolytic inhibitors. This latter statement is in contradistinction with the studies of several authors [3, 11, 12, 17] indicating that the fibrinolysis in cirrhosis may also be related to decreased serum plasmin inhibitors.

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This discordance prompted us to study both hypotheses in a group of 25 cirrhotic patients in different stages of evolution of the disease. The present investigation involved the assay of various fibrinolytic parameters before and after activation of the fibrinolytic system by nicotinic acid.

Materials and Methods

Reagents. Saline (0.07 M)-phosphate (0.06 M) buffer pH 7.5 (S.P buffer) [10]. – Glycerol-phosphate buffer containing equal volumes of glycerol and S.P buffer. – Fibrinogen Bovin (Poviet, Amsterdam) *lot 57* (protein 78.2%, clottable fibrinogen 62.5%, NaCl 14.9%) dissolved freshly before use, in concentration of 25 mg/7 ml S.P buffer. – Thrombin (Topostasine Roche) 20 NIH units per ml S.P buffer stored at 20° C. – Plasmin: chloroform activated bovine fibrinolysin (Parke, Davis and Co.) 1,300 Loomis units/vial, diluted in 650 ml glycerol-phosphate buffer and stored at –20° C, is diluted adequately with the same solvent before use so that 0.10 ml plasmin lyses the standard clot (formed by mixing 0.35 ml S.P buffer, 0.25 ml fibrinogen solution and 0.10 ml thrombin) in a 9–11 minute range. This plasmin working solution is kept on ice *during* the whole procedure and discarded when the lysis time of the standard clot exceeds 11 min.

Fibrinolytic activity was measured in the plasma and in the euglobulin fraction, on unheated bovine fibrin plates, prepared according to ASTRUP and MÜLLERTZ [1]. For statistical evaluation the individual values of lysed surface were converted into fibrinolytic activity by reading on linear reference curve constructed from plots of log lysed surface on bovine fibrin plate versus log sample concentration. This reference curve was obtained from pool of fibrinolytic active euglobulin precipitates, reconstituted with veronal buffer into number of "activator" levels. Similar curves for individual cirrhotic precipitates showed slopes comparable to that of the reference curve.

Antiplasmin assay (according to JOHNSON [10] with slight modifications) 0.1 ml of serum dilutions (1/25, 1/50 and 1/100) in S.P buffer are transferred to a testtube at room temperature. 0.15 ml of the plasmin working solution is added. The tubes are mixed gently and kept at room temperature for 10 min.

Consecutively are added S.P buffer 0.40 ml, fibrinogen 0.25 ml, thrombin 0.10 ml. *Immediately after adding thrombin, a time reading device is started, the reagents are* mixed by mechanical swirling for about 10 sec and the tube is transferred to a water bath at 37° C. Along with each series of serum dilutions reference clot is made containing 0.10 ml plasmin dilution and buffer up to 0.65 ml. Fibrinolytic endpoints, indicated by the rising of all air bubbles into the upper half of the test mixture, are read visually or in the lysis *recorder* [4]. All determinations are performed at least in duplicate. The antiplasmin content of the serum is calculated from a plot on log-log paper the lysis times being plotted against the reciprocal of the final serum concentrations. A line is drawn joining the two dots with lysis times adjacent to the reference time. On this line the serum dilution, producing lysis time equal to the reference time is looked for. As 0.10 ml plasmin dilution contains per *definition 1 plasmin* unit, *the reciprocal of the serum concentration which prolongs the* lysis time of 0.15 ml plasmin to the time of 0.10 ml plasmin (reference time) inhibits 0.5 plasmin units. Divided by two, this reciprocal of the serum concentration gives conventionally the antiplasmin content of the sample.

Experimental groups. Nicotinic acid was administered in two series of 25 subjects. The cirrhotic *group consisted* of 25 hospitalized patients, in whom the diagnosis of hepatic cirrhosis, in different stages of evolution was documented by physical examination, laboratory findings, laparoscopy and proved in all of them by liver biopsy- none of the patients was taking corticosteroids, but some were under intermittent *diuretic* treatment. The

control group consisted of 25 laboratory personal and hospitalized psychosomatic patients without any known physical or biochemical abnormality. Mean age (controls 48 years; cirrhotics 50 years), mean body weight (controls 63 kg; cirrhotics 66 kg), mean cholesterol value (controls 238 mg%; cirrhotics 208 mg%), mean hemoglobin value (controls 14 g%; cirrhotics 13.2 g%) and sex ratio (3 males, 1 female) were comparable. Nicotinic acid (100 mg) was rapidly injected *i.v.* to the recumbent person in the mid-afternoon. Under these conditions, diurnal changes were assumed to operate uniformly [6].

Venous blood was sampled just before and 15, 30 and 60 min after the *i.v.* administration. The tourniquet was applied during about 15 sec before puncture of an antecubital vein. The blood was collected directly in glass tubes for preparation of serum and in precooled siliconized tubes, containing potassium oxalate (H_2O) 2.5% (1 part for 9 parts of blood) for fibrinolytic determinations. Serum was pipetted off after clotting of the whole blood for 2 h at 37°C. After centrifugation at 4 °C for 10 min and 4,000 *g* the plasma and the serum were stored in polystyrene tubes at −20°C until used. Serum from 20 healthy donors was obtained in a similar manner and mixed in equal proportions for constituting a serum pool, used for preliminary investigation of the reproducibility of the antiplasmin assay.

Results

Determination of *in vivo* Plasminogen Activator Clearance

The fibrinolytic activity in the euglobulin fraction, measured on unheated bovine fibrin plates, will be considered according to SHERRY *et al.* as blood plasminogen activator activity [20]. Figure 1 shows the individual values of euglobulin fibrinolytic activity before (sample 0) and 15 min (sample 1), 30 min (sample 2) and 60 min (sample 3) after nicotinic acid administration.

As there is no linear correlation between lysed surface and activator

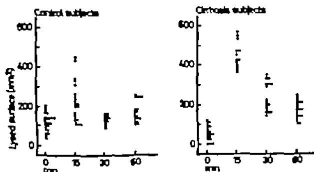


Fig. 1. Euglobulin fibrinolytic activity measured on bovine fibrin plates before and 15, 30 and 60 min after intravenous injection of 100 mg nicotinic acid, in control subjects and cirrhotic patients.

content, it is necessary for statistical analysis to compute these lysed surfaces shown in figure 1 into activator concentration by reading from a linear reference curve (plotting log mm² lysed surface versus log activator activity) After calculation of the mean activator activity in the different samples of both groups these mean data are expressed in percentage of the mean activator activity in the control group before nicotinic acid injection. These calculated mean values are for blood samples 0 1 2 and 3 respectively 100 206, 152 and 137% for control subjects and 66, 405 274 and 154% for cirrhotic patients The lower mean value found in the cirrhotic patients, before nicotinic acid injection, is not statistically different from the mean control value ($0.05 < P < 0.10$) The increase in euglobulin fibrinolytic activity from sample 0 to sample 1 is statistically significant for both series ($P < 0.001$) the mean increase being higher for the cirrhotic patients in comparison to the control subjects ($P < 0.001$) After the 15 minute sample, the euglobulin lytic activity declined progressively to base-line values, more rapidly for the control subjects than for the cirrhotic patients.

As the in vivo disappearance rate of plasminogen activator is an exponential function of activator concentration with respect to time [7] the 50% in vivo decay time of euglobulin fibrinolytic activity can be calculated from the formula $\log Y = \log A + bx$ and $T\ 1/2 = \log 2/b$ The calculated mean in vivo 50% disappearance time of activator is 13.8 min for those control subjects who clearly responded to nicotinic acid injection and 24.5 min for the total group of 25 cirrhotic patients ($0.002 < P < 0.01$) The 50% in vivo activator decay is 26.2 min for the 10 cirrhotic patients exhibiting the most serious coagulation abnormalities and clinical signs of severe hepatic decompensation. These findings indicate that the in vivo disappearance of plasminogen activator in the cirrhotic patients is significantly longer compared to that of the control subjects.

Antiplasmin Determination

The reproducibility of the antiplasmin assay has been preliminarily investigated. The mean of normal pool values, obtained over several weeks, is 360 antiplasmin units per ml serum, with a standard deviation of 25 units. Individual normal values vary from 210–510 units per ml, $\bar{x} = 344$ $s = 94$ and $n = 20$

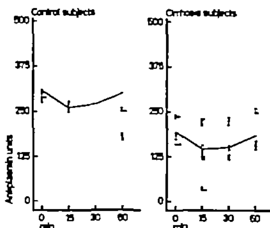


Fig. 2. Antiplasmin activity before and 15, 30 and 60 min after intravenous injection of 100 mg nicotinic acid in control subjects and cirrhotic patients.

The serum antiplasmin activity has been assayed before and after nicotinic acid injection in both experimental groups. The individual experimental results are summarized in figure 2, the mean values being for blood sample 0 1 2 and 3 respectively 311 263 271 and 296 units for the control persons and 192, 145 153 and 169 units for the cirrhotic patients. The mean antiplasmin content before fibrinolytic activation in the cirrhotic patients is significantly lower than in the control group ($P < 0.001$) Activation of the fibrinolytic system by nicotinic acid is associated in the 15 min blood sample with a decrease in serum antiplasmin content for both series, which is highly significant ($P < 0.001$) when evaluated with the method of paired comparison. The decrease in serum antiplasmin is maximal at the moment of maximal increase of euglobulin fibrinolytic activity Subsequently the antiplasmin capacity rises steadily towards initial values, in association with a progressive decrease in euglobulin fibrinolytic activity

In order to evaluate the importance of the decreased antiplasmin level in liver cirrhosis, a comparison was made between the fibrinolytic activity on fibrin plate in total cirrhotic plasma samples versus control plasma samples both with an identical *fibrinolytic activity* in the corresponding euglobulin precipitates. Indeed, the difference between plasma and euglobulin lytic activity is mainly based upon the elimination of the bulk of inhibitors (the still hypothetical anti-activator and the antiplasmins) in the euglobulin fraction. After plotting the various

plasma fibrinolytic activities versus the corresponding euglobulin fibrinolytic activities for both series, the correlation coefficient b between the plasma and euglobulin lytic activity measured on bovine fibrin plate, was calculated as 0 650 for cirrhotic patients and 0.268 for control subjects. The corresponding difference in slope between both regression lines is statistically significant ($0.02 < P < 0.05$) confirming the importance of the decrease in fibrinolytic inhibitors in explaining the susceptibility of the cirrhotic patients for induction of fibrinolytic activity in total plasma.

Discussion

Various observers have reported increased fibrinolytic activity in liver cirrhosis, measured by the autodigestion time of clots of total or diluted blood or plasma of the patient. In our experiments, the lytic activity before nicotinic acid injection was measured on a standard lysable fibrin substrate, not variable from patient to patient, and no evidence for enhanced plasminogen activator activity in the euglobulin fraction of 25 cirrhotic plasma samples was obtained (fig. 1) Nevertheless, patients with liver cirrhosis are prone to develop increased fibrinolytic activity in certain circumstances [8 9 19] Many authors relate the increased fibrinolytic activity to the release of an increased amount of plasminogen activator in the cirrhotic plasma.

Our data confirm the known susceptibility of the patients with liver cirrhosis to develop increased fibrinolytic activity after stimulation of the fibrinolytic system (fig 1) According to FLETCHER *et al.* [7] a major factor in the abnormal fibrinolytic response to nicotinic acid, is a failure of hepatic clearance mechanisms for plasminogen activator in liver cirrhosis. Calculating the activator concentration by assessment of the euglobulin lysis time, these authors find a mean in vivo 50% plasma activator clearance rate of 13 ± 5 min in control persons versus 53 ± 19 min in cirrhotic patients.

In our experiments blood plasminogen activator precipitated almost quantitatively in the euglobulin fraction, is measured on bovine fibrin plates. From our data the calculated mean in vivo 50% disappearance time of activator activity is 13.8 min for the control subjects, who clearly responded to nicotinic acid injection, and 24.5 min for the total group of 25 cirrhotic patients ($0.002 < P < 0.01$) Notwithstanding the methods in both studies differ the in vivo disappearance of plasminogen activator in the two control series is

strikingly similar However the prolongation of the *in vivo* decay of plasminogen activator in cirrhotic patients in comparison with control subjects is less pronounced in our study As differences in patient material (our patients being scattered over a wider range of liver insufficiency) could provide a possible explanation for this discrepancy we calculated the *in vivo* plasminogen decay in the 10 cirrhotic patients with the most seriously disturbed coagulation set and clinical signs of severe liver failure, but the 50% plasminogen decay time of 26.2 min in this group was still the half of the value found by FLETCHER [7] The significantly longer *in vivo* disappearance time of plasminogen activator in cirrhotic patients, compared to control subjects, is according to FLETCHER [7] ascribed to insufficient hepatic clearance mechanisms of activator by the diseased liver However it has to be kept in mind that other still hypothetical mechanisms may be involved in explaining the differences in activator disappearance time after nicotinic acid injection, such as differences in possible renal clearing of activator activity differences in continuous activator release during the declining part of the disappearance curve, differences in nicotinic acid metabolism, and differences in level and rate of release of anti blood activator activity The availability of purified blood plasminogen activator is required for exact activator clearance studies and for further elucidation of the mentioned hypothetical mechanisms.

The role of the fibrinolytic inhibitors in the genesis of the increased fibrinolytic activity in liver cirrhosis and the enhanced susceptibility to stimuli capable of inducing fibrinolytic activity is debated. According to FLETCHER *et al.* [7] assays of plasma antiplasmin, performed with a caseinolytic technique, revealed no significant difference between the normal and cirrhotic group. Studies of other authors however [3 11 12, 17] indicate that the fibrinolytic activity in liver cirrhosis may be related to a decrease in the plasmin inhibitors in serum, this finding being in agreement with the previously stated lowered levels of alpha$_2$-globulins in cirrhosis [18] We performed a serum antiplasmin assay in 25 cirrhotic patients according to the fibrin clot digestion method as proposed by JOHNSON [10] Interference in the test by residual activator activity in the serum samples was considered as negligible if not inexistent both for reasons of prolonged clotting at 37°C of the blood and subsequent extensive dilution (250–1 000 times) of the serum sample. As autodestruction of plasmin occurs during the test, the error may be minimized by measuring serum inhibition after a relatively short incubation time (10 min) and by adding glycerol as a

stabilizer to the plasmin solution. Although the plasmin inhibition is not fully evolved this short preincubation time may be reliable as the bulk of serum antiplasmin activity resides in the alpha$_2$-globulin fraction, which produces an immediate temperature independent inhibition of plasmin [13 15 16] The mean antiplasmin content (192 U/ml serum) in the cirrhotic patients is substantially lower than the corresponding value in control subjects (311 U/ml serum) this difference being highly significant ($P < 0.001$) (fig 2) The importance of the decrease in fibrinolytic inhibitors (the still hypothetical anti activator and the antiplasmins) was evaluated by comparing the lytic activity in cirrhotic versus control plasma samples with an identical corresponding lytic activity in the euglobulin fraction where the bulk of fibrinolytic inhibitors is eliminated. Our finding of a significantly higher plasma fibrinolytic activity in cirrhotic plasma in comparison with control plasma for an identical euglobulin lytic activity supports the hypothesis that the susceptibility of the cirrhotic patients for induction of fibrinolytic activity in total plasma may be related to decrease in fibrinolytic inhibitors ($p < 0.05$) Decreased antiplasmin levels in cirrhosis are probably not only due to decreased hepatic synthesis but also to increased consumption in plasmin antiplasmin complex formation. Indeed it has been demonstrated that plasmin-antiplasmin complex may form a circulating reservoir of plasmin which is innocuous to other circulating proteins but forms a ready source of plasmin when required for the lysis of fibrin. The extent of such plasmin binding may be appreciated from the work of Nanninga and Guest [14] who have shown that bound plasmin is present in a hundredfold greater concentration than free plasmin at a plasmin concentration of 10^{-7} M.

There is a suspicion that not only the antiplasmin content but also the level of the still hypothetical inhibitor of blood plasminogen activator is decreased in liver cirrhosis. As it has repeatedly been shown that increased fibrinolytic activity is frequently the result of the presence of an activator of plasminogen and not of plasmin an inhibitor of this activator may have important physiological implications. However it has until now been impossible to measure such an inhibitor as the activator itself has not been isolated and purified.

Summary

Patients with liver cirrhosis are prone to develop increased fibrinolytic activity after i.v. injection of nicotinic acid. This abnormal response is due to failure of hepatic clearing of plasminogen activator. The calculated mean in vivo 50% disappearance time of activator activity is 13.8 min for 25 control subjects and 24.5 min for 25 cirrhotic patients ($0.002 < p < 0.01$). Decreased antiplasmin levels in cirrhotics may potentiate the susceptibility of these patients to develop plasma fibrinolytic activity. This difference between the mean antiplasmin content (192 U/ml serum) in the cirrhotic patients and the corresponding value in the control subjects (311 U/ml serum) is highly significant. The decrease in fibrinolytic inhibitors is probably related with the finding of significantly higher fibrinolytic activity in total cirrhotic plasma in comparison with control plasma for an identical level of plasminogen activator activity in the corresponding euglobulin fraction of both cirrhotic and normal plasma ($0.02 < p < 0.05$).

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A Specific Inhibitor of Human Clotting Factor V

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In 1958 Ferguson *et al.* [5] reported on a patient suffering from a heavy haemorrhagic diathesis due to the presence of an inhibitor of factor V. This inhibitor showed no species-specificity since it was found to inhibit bovine factor V as well. The therapeutic effect of ACTH suggested that this anticoagulant could have been of immunological origin although no direct proof for this assumption was offered.

We have observed a similar coagulation disorder in a 66-year-old woman. An inhibitor which was effective only against human factor V could be demonstrated in the plasma as well as in the serum of the patient.

Case Report

M.R.L. 1899, was admitted to the hospital on September 26th, 1965 because of right-side pleurisy. Prior to hospital admission she had been treated with chloromycetin, Tanderil® and Lederkyn®. From her history it became clear that she had suffered from specific (tbc) lung infiltration already 40 years ago.

After few days in the hospital the patient developed pericarditis. Tuberculostatic therapy was started. The treatment of thrombophlebitis of the right leg which developed early in 1965, was followed by prolonged Butazolidin® therapy. The patient' condition had improved significantly by November 15th, 1965 and she was discharged from the hospital; however 6 days later the patient had to be readmitted as an emergency with haematuria which had persisted for 2 days already. On the medial side of the right thigh and on the abdomen small to medium-size suffusions were visible. The patient was afebrile. A cystoscopy gave no evidence for mucous membrane lesions.

Laboratory findings: haemoglobin 54%, haematocrit 21%, bleeding time 3½ min, clotting time 5 min, thromboplastin time determined according to Quick 33%, thrombocytes 136,000/mm³.

The patient received blood transfusions; in spite of serological compatibility she developed temperature of 39.2° C after the fourth transfusion.

An analysis of the blood clotting system was performed on blood sample received on November 23rd, 1965 (table I). The most striking result consists in the pronounced reduc-

tion of factor V activity as measured by the one stage assay. Correspondingly the global tests of the intrinsic system (recalcification time, hirudin tolerance time, partial thromboplastin time and prothrombin consumption test) as well as thromboplastin time were all pathological. The moderately diminished factor XI activity was probably due to the low factor V content of the factor XI-substrate used.

The patient's history gave no indication of parahaemophilia: she never had bleeding episodes before, although she was allergic to penicillin. Furthermore, hereditary coagulopathy seemed excluded by the absence of bleeding disorders in the patient's family. Thus, the presence of an inhibitor of factor V appeared probable.

The patient's blood was reexamined on December 4th, 1965 (table I). Factor V activity was still markedly reduced, and hyperfibrinogenaemia had developed.

A search for auto- and iso-antibodies against red cells, for leucoagglutinins, for cell nucleus antibodies, for rheumatoid factor activity and for antibodies against immunoglobulins gave negative results.

Special Investigations

1. Substitution experiments in Quick's thromboplastin test. The following incubation mixture was used: 0.1 ml citrated plasma (patient's and normal pooled plasma mixed in different ratios), 0.1 ml thromboplastin 'Roche', 0.1 ml 0.4 M $CaCl_2$. The results of substitution experiments using the patient's plasma (M-plasma) and normal plasma are shown in figure 1.

In a second series, cross-correction experiments were performed using different incubation times of the patient's plasma with normal plasma. In these experiments the plasma was diluted *1:10* with isotonic barbital buffer (figure 2). The results obtained must be interpreted in terms of the presence of an anticoagulant inhibiting the prothrombin-conversion by the extrinsic activation mechanism. The activity of this anticoagulant appeared immediately but increased on prolonged preincubation.

2. Effect of the inhibitor on thromboplastin generation. As shown in figure 3, the thromboplastin generation test (TGT) [3], in which $BaSO_4$-absorbed patient's plasma and normal

Table I. Clotting investigations

	25-11-65	4-12-65	5-1-66	Normal range
Recalcification time, sec	450	840	115	90-180
Hirudin tolerance time, sec	570	1,200	120	~ 300
Partial thromboplastin time (Asolectin), sec	300	510	95	70-180
Prothrombin consumption test, %	45	35	5	0-8
Thromboplastin time, sec	55	65	12	~ 15
Thrombin time, sec	11	16	14	~ 10
Fibrinogen (Schulz), mg %	400	600	300	~ 300
Factor II activity, sec	19	17	18	~ 19
Factor V activity, %	5	3	60	70-120
Factor VII activity, %	90		100	70-120
Factor VIII activity, %	100	100	100	70-120
Factor IX activity, %	100	100	–	70-120
Factor X activity, %	100	–	100	70-120
Factor XI activity, %	22	–		70-120

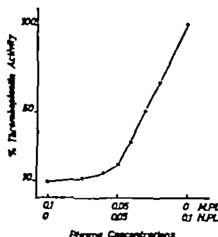


Fig 1 Substitution experiments in Quick's thromboplastin test. *M.Pl.* patient's plasma, *N.Pl.* normal plasma.

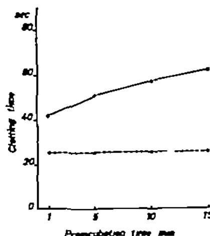


Fig.2. Cross correcting experiments with patient's plasma and normal plasma t different preincubation times. ● —— ● mixture of patient plasma and normal plasma; ● —— ● normal plasma alone.

serum were used, is also influenced by the inhibitor This experiment shows, furthermore, that the inhibitor is not or only poorly absorbed by BaSO I proved necessary to modify the standard conditions of the TGT whereby concentrated reagents ($BaSO_4$-absorbed plasma and serum) were used in order to demonstrat the activity of the inhibitor

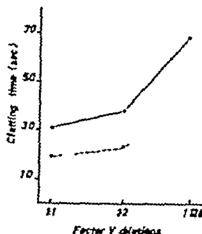


Fig. 7 Activity of human and bovine factor V preparations. ●——● human factor V preparation ●——● bovine factor V preparation.

shown in figure 5. They appear to demonstrate the presence of an anticoagulant directed against factor V. These findings also confirm that the inhibitor is present in both plasma and serum, and that it is effective upon incubation.

Patient's serum and normal plasma were preincubated for different periods of time in order to establish the interdependence of the inhibitory effect and preincubation time: the one-stage factor V assay was used. The results represented in figure 6 demonstrate that the inhibitory effect increases correspondingly with increasing incubation times.

4. Inhibition experiments with factor V-preparations of bovine and human origin. Factor V was prepared according to a slightly modified version of OWREN's method [15]. The activity of the lyophilised product was tested as follows: 25 mg of factor V preparation of bovine origin and 100 mg factor V preparation of human origin, respectively, were dissolved in 1 ml barbital buffer. Factor V activity was determined in dilutions prepared from these solutions. Both preparations were tested for activity of other clotting factors: only factor V activity could be demonstrated.

For demonstration of inhibitor activity both, bovine and human factor V-preparations were adjusted to equal activity, as based on their respective activity curves (figure 7). 0.5 ml factor V-preparation (human or bovine) was preincubated with 0.05 ml of the patient's plasma for different lengths of time: the clotting activity was measured by determination of the thromboplastin time. As a control, the patient's plasma was substituted by factor V reagents. Results are shown in figure 8, demonstrating that the inhibitor is specifically directed against human factor V but not effective against bovine factor V. Furthermore, it appears from the shape of the curve that both preparations may have lost some of their activity during the test procedure.

5. Stability of the anticoagulant. The inhibitor showed no loss of activity after standing at +4°C or −20°C for several weeks. Incubation at 37°C for 24 h likewise caused no loss of activity. After 1 h at 60°C no decrease of activity was discernible, whereas at 70°C the activity disappeared after 30 min.

6. Chemical and physicochemical properties of the anticoagulant. The inhibitor was neither absorbable on aluminium-hydroxide nor on $BaSO_4$ [2]. It is not lipophilic since after shaking with ether it was still found in the water phase.

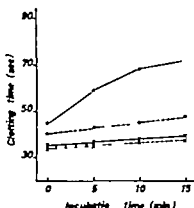


Fig. 8. Dependence of the inhibitor's activity against factor V preparations on preincubation time

●——● human factor V preparation + patient plasma
●– – –● human factor V preparation + factor V deficient plasma
×——× bovine factor V preparation + patient's plasma
×– – –× bovine factor V preparation + factor V deficient plasma

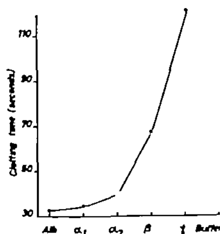


Fig. 9. Distribution of the inhibitor activity among electrophoretic fractions of the patient's serum.

In order to determine the electrophoretic mobility of the inhibitor 0.1 ml of the patient serum was applied to each of 13 paper strips. After electrophoresis, one strip was colored with amido black to localise the different fractions, and the 5 main electrophoretic fractions thus localized were cut out in the remaining 12 strips. The paper cuttings were eluted during 12 h in barbital buffer at 4 C, whereby the amount of barbital buffer added was proportional to the protein content of the different fractions. Of each eluate 0.3 ml were

mixed with 0.1 ml of normal plasma, diluted 1:10 with isotonic barbital buffer during 30 min. Factor V activity was then assayed by the one-stage test. Figure 9 shows that the inhibitory activity was contained mainly in the γ-globulin fraction.

7. *Immunochemical investigations.* The patient's serum was submitted to OUCHTERLONY immunodiffusion tests against human and bovine factor V-preparations, against normal pooled plasma as well as against a factor V-free plasma (factor V reagent). A weak but definite precipitation line formed between the patient's serum and human factor V preparation, whereas reaction of the patient's serum with normal plasma pool remained doubtful. No reaction was observed between the patient's serum and bovine factor V preparation, and no precipitation line was formed, as expected, between the patient's serum and factor V-free plasma. Due to the weak appearance of the precipitation line it was not possible to obtain convincing photographic picture for reproduction.

Follow-up Studies

On December 18th 1965, a new sample of citrated blood was tested. No alteration in the clotting defect was discernible. Factor V activity was still only 5% of normal values.

The patient's general condition was unchanged. After transfusion haematuria stopped for a few days, but then bleeding resumed. On December 20th, 1965, ACTH-therapy was started. A daily dose of 25 mg was given until January 8th, 1966. A few days after initiation of this therapy the general condition improved both objectively and subjectively; haematuria stopped and the thromboplastin time showed substantial improvement amounting to 60% by December 29th, 1965. On January 5th, 1966, the coagulation analysis was reassessed. With the exception of a slightly reduced factor V activity (60% of normal values) blood coagulation appeared normal (table I). On January 9th, 1966, the patient went to the bathroom where she suddenly collapsed and died. An embolic obstruction of the pulmonary artery as well as thromboses of the left femoral and iliacal veins were revealed at autopsy.

Discussion

The appearance of haemorrhagic diathesis caused by the action of inhibitors of the clotting system is well known. This phenomenon may be due to either a raised activity of physiological inhibitors (e.g. antithrombins) or the appearance of (usually acquired) circulating anticoagulants (e.g. specific inhibitors of factor VIII). Circulating anticoagulants are most frequently directed against factors VIII and IX or against tissue thromboplastin; however specific inhibitors of most other clotting factors may be found as well [8].

Formation of circulating anticoagulants specifically directed against factor V appears to be very rare. In addition to the one case mentioned by FERGUSON *et al.* [5] there exists only the observation reported by HÖRDER [9, 10] in 1955. A critical evaluation of HÖRDER's case especially with regard to the genetic pattern of the haemorrhagic diathesis and to his interpretation of the experimental data leaves it very doubtful that a factor V inhibitor was present at all. We are inclined to agree with FERGUSON *et al.* who concluded that HÖRDER described a case of parahaemophilia.

The case reported in this study is very similar in its characteristics to the one described by FERGUSON. As a result of the inhibition of factor V an impairment of the prothrombin-conversion by the intrinsic as well as by the extrinsic activation mechanisms occurs accordingly the recalcification time the hirudin tolerance time, the partial thromboplastin time, as well as the thromboplastin time are distinctly prolonged. Inhibition experiments with purified factor V preparations clearly demonstrate the factor V specificity of the inhibitor. However the inhibitor present in our case, contrary to the inhibitor of FERGUSON *et al.*, did not react with bovine factor V and therefore appears to be species-specific. Furthermore it may be concluded from inhibition experiments, that the inhibitor is exhibiting immediate as well as progressive activity. Evidence indicating that the inhibitor could be an antibody appears to be of particular interest. This concept is supported by the physicochemical properties (electrophoretic behaviour as a γ-globulin) by the effect of ACTH-therapy but mainly by the results obtained in immunochemical analyses. In fact the inhibitor behaved as a weak precipitin it must be considered, however that most precipitating antibodies form only after prolonged and powerful antigenic stimulation.

For a long time, most authors were inclined to believe that acquired circulating anticoagulants were antibodies [8]. Inhibitors found in multi-transfused haemophiliacs are classified as isoantibodies since these patients do not possess the corresponding antigens (factor VIII or factor IX, respectively). Inhibitors observed *post partum* often can be related to an iso-immunization in this case, one must postulate group-specific differences in the antigenic structure of the clotting factor in question. However such differences have not as yet been demonstrated.

Inhibitors detectable in cases of collagen disease (e.g. in patients with *lupus erythematodes disseminatus*) but also during the course of other diseases, such as chronic glomerulonephritis, liver cirrhosis, tuberculous lymphadenitis, sepsis lenta, lues, pemphigus vulgaris, dermatitis herpetiformis, are likewise attributed to an immunologic origin. In all these cases, an autosensitization must be assumed. Indirect support for the concept of the antibody character of circulating anticoagulants in most instances may be gained from the patient's history or from the physicochemical behaviour of the inhibitor. Direct serological evidence has been presented only in a few cases occasionally precipitating antibodies against factor VIII or factor IX could be demonstrated

[4, 6, 11, 12, 18, 19]; in other cases the inhibitor activity could be removed with a specific anti-immunoglobulin anti serum [2, 16, 1, 7]. To our knowledge, an inhibitor with antibody activity directed against factor V has never been demonstrated before. In our case, the antibody may be of autoimmune origin, although the question of aetiology has to remain open. Neither clinical nor serological evidence for a generalised autoimmune disease was found. The aetiological role of drugs in the induction of autoantibody formation has to be considered. As mentioned already, the patient was allergic to penicillin. STEFANINI and DAMESHEK [17] reported on a patient with purpura SCHÖNLEIN-HENOCH, who developed a circulating anticoagulant directed against factor VIII after the administration of penicillin procaine; simultaneously the clinical picture deteriorated. Dysproteinaemia of the γ-globulin type observed in this patient was traced back by the authors to the presence of large amounts of the inhibitor, although no direct proof for its antibody character was offered. An analoguous case has also been described by NUSSEY and DAWSON [13].

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We are greatly indebted to Dr. H. VORZELL, Langnau, for putting blood samples as well as the case history and clinical data of his patient at our disposal.

Summary

A severe haemorrhagic diathesis in a 66-year-old woman was found to be due to a circulating anticoagulant directed against clotting factor V. The inhibitor was species-specific and active immediately. Immunochemical studies presented evidence for the antibody character of the inhibitor.

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Another Family Showing the Interaction of the Genes for Hb G and Hb S[1]

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The combination of haemoglobin Gα and haemoglobin Sβ is rare. Pootr et al. [12] described a family in which this abnormality resulted in 4 major haemoglobins in one individual and in mild anaemia. To our knowledge, no other instance of Hb Gα Hb Sβ combination has so far been reported. More frequent are instances of Hb Gα combined with Hb Cβ, which also lead to the presence of 4 haemoglobin components and to mild haematological symptoms. Smith and Torbert [18] described 4 haemoglobin components in cases of Hb Hopkins 2 – Hb S and Hall Craggs et al. [5] in cases of Hb S – Hb Stanleyville II. Hb Hopkins 2 and Hb Sta II are also abnormal in the α-chains. Family studies in such cases revealed that the mutants of the α- and β-chains were inherited independently of one another. The study of such exceptional families is essential to understand the mode of inheritance of haemoglobin abnormalities and to obtain a better idea of the arrangement of the genetic loci in the chromosomes. The more families that are studied, the better one can calculate the probable relative location of the genes.

We therefore believe it important to report another case of Hb Gα combined with Hb Sβ. It provides more data on the clinical and haematological expression of such an interaction and on the chromo-

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somal relationship of the genes responsible for abnormal haemoglobins in an additional family

Methods

Haematologic examinations were conducted according to standard methods. Haemoglobin electrophoresis was performed on starch gel [19] and on agar gel [15]. Alkali denaturation of haemoglobin followed the method of SINGER *et al.* [17] with 2% of the total amount of haemoglobin taken as the upper limit of normal. The solubility test was performed by the method of ITANO [9]. Haemoglobin components were quantitatively analyzed on diethylaminoethyl (DEAE) cellulose column chromatography by the method of HUISMAN and DOZY [7]. Polypeptide chains of haemoglobin were studied on starch gel electrophoresis in an urea-barbital system at pH 8.0 [4]. Fingerprinting of peptides was performed by BAGLIONI's [2] modification of INGRAM's method [8].

Isolation of peptides. Peptides for amino acid analysis were isolated on Whatmann 3MM filter paper by one-dimensional electrophoresis followed by descending chromatography as described by INGRAM [8]. Additional purification was achieved by descending chromatography in butanol, acetic acid, and water solvent (4 1 5 upper layer 24 h). After each step of separation, guide strips were cut from both sides of the paper and dipped in ninhydrin to locate the position of the band containing the peptide to be isolated. This region was then eluted with solvent of ethanol, acetic acid and water (50 0.15 49.85) pH 3.8, and the eluate was evaporated to dryness in desiccator.

Amino acid analysis. The isolated peptides were hydrolyzed in 6 N HCL under reduced pressure at 110° C for 20 h, and the amino acids were analyzed on Spinco automatic amino acid analyser (Model 120 C).

Case Report

H. E., the propositus, was 45-year-old Negro man who complained of prolonged tiredness. Upon examination he was somewhat pale but nonicteric and otherwise physically normal, without enlarged spleen or liver. Repeated blood examinations revealed mild anaemia with slight microcytosis. Haematological findings, when the patient was examined again on September 21 1967 after repeated iron therapy were as follows: Hb 12.3 g per 100 ml, RBC 5.40 million/mm³, PVC 39.0%, MCV 72.2 μm³, MCH 22.8 pg, MCHC 31.5 %, reticulocytes 2.1%. WBC and platelet counts normal. The peripheral blood smear showed slight anisocytosis and poikilocytosis with few target cells. Sickling of the erythrocytes took place, although not readily when they were deprived of oxygen. Alkali-resistant haemoglobin was 0.5% of the total amount of haemoglobin. Acid elution for Hb F was negative. Serum bilirubin was not increased. Haemoglobin electrophoresis revealed 4 components (1) Hb A, (2) haemoglobin component with the mobility of Hb S, (3) component with the mobility of $Hb\ A_2$, and (4) slower haemoglobin component more cathodic than $Hb\ A_2$ and resembling $Hb\ B_2$ (fig. 1).

Examination of the patient's wife, 2 daughters, and 3 sons disclosed the following: S. E., the 32-year-old Negro wife, was physically and haematologically normal. Her haemoglobin electrophoretic pattern was also normal, she showed no increase of fetal haemoglobin and her $Hb\ A_2$ level was not elevated. The 2 daughters, Qd and Qt, 15 and 14 years old, were physically normal. Their peripheral blood showed slight microcytosis, anisocytosis, and poikilocytosis and contained few target cells. Results of the sickling test were positive, but sickling did not take place readily. The pattern of haemoglobin electrophoresis in these 2 daughters was identical to the father's, again consisting of 4 haemoglobin components

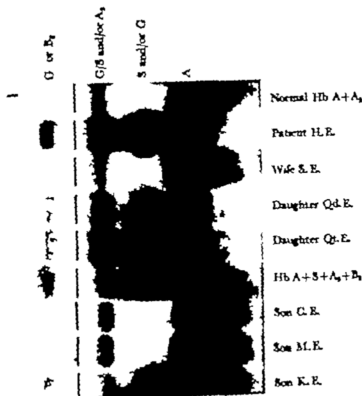


Fig. 1. Starch gel electrophoresis in tris-EDTA-boric acid buffer pH 8.6 of the haemolysates of patient and family.

(fig. 1). Hb F was not increased. All 3 sons were physically normal. C. E., the eldest, 17 years old, was haematologically normal and had normal haemoglobin pattern on electrophoresis, normal Hb A_2 level, and no increase of Hb F. K.E., the 11 year-old second son had a haemoglobin pattern like that of the father and two sisters. It consisted of 4 haemoglobin components, but their quantitative distribution differed (see 'Further Haemoglobin Studies') and his erythrocytes did not sickle. M. E., the 10-year-old youngest son had normal electrophoretic haemoglobin pattern without increase of Hb F or Hb A_2. The haematological findings in all members of the family are listed in table I. After prolonged oral iron therapy haematological studies were repeated in all members of the family but they were found to be essentially unchanged.

Further Haemoglobin Studies

Electrophoresis. Haemoglobin findings on starch gel electrophoresis have already been presented (Case Report). On agar gel electrophoresis, the haemolysate of the patient and the 2 daughters, whose red blood cells

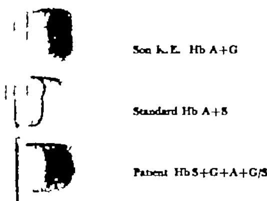


Fig. 2. Agar gel electrophoresis in citric acid-sodium citrate buffer pH 6.2 of patient haemolysate compared with that of his son K. E. and standard Hb A + S. Note that there is no separation in the haemolysate of son K. E.

showed sickling, revealed two clearly separated components, the slower-moving one having the same mobility as a standard Hb S (fig 2) This slower component was smaller than the component with the mobility of Hb A on agar gel electrophoresis, contrary to the finding on starch gel electrophoresis. The wife and 3 sons, including K. E., who showed a component like Hb S on starch gel electrophoresis but whose erythrocytes did not sickle, each had just one band like normal Hb A, indicating that in K. E. the slow moving Hb S like component, on starch gel electrophoresis was not Hb S This explains the inability of his erythrocytes to sickle

Column chromatography Quantitative analysis of haemoglobin components of family members was made by column chromatography These components in the propositus were Hb A 39.5% the component with the mobility of Hb S 43 9 % the one with the mobility of Hb A_2 14 6% and the slowest component 2% Percentages for the same components in the eldest daughter were 37 0 46.8 14.8 and 1 4% respectively Insufficient blood was obtained from the second daughter to perform quantitative analysis on column chromatography Data for K. E., with a component like Hb S on starch gel electrophoresis, were Hb A 70% the component like Hb S 26.9% Hb A_2 2 and the slowest component 1 1% To further demonstrate that the component like Hb S in this son was not Hb S we separated and purified it by column chromatography A solubility test showed that this component, unlike solutions containing Hb S was fully

Table I Haematological data in patient and family members

	Sex	Age years	Hb g/100 ml	RBC 10^6/mm	Hct %	MCV μ^3	MCH pg	MCHC %	Reti-culo-cytes %	Hb A %	Hb S or Hb G %	Hb G/S[1] and Hb A_2 %	HbA_2 %	HbO_2 %	Hb alk. resis-tant %
H. E., propositus	M	45	11.8	3.06	38.0	75.1	23.3	30.5	2.1	30.3	43.9	14.8		2.0	0.5
S. E., wife	F	52	13.6	4.80	45.0	89.6	28.3	31.6	1.4	87.6	0	–	2.4	0	1.0
C. E., son	M	17	12.7	4.95	41.0	82.8	25.7	31.0	0.3	97.5	0		2.5	0	1.5
Qd. E., daughter	F	15	12.2	3.25	36.0	68.6	23.2	33.9	1.6	37.0	46.8	14.8	–	1.4	0.8
Qt. E., daughter	F	14	12.6	5.70	38.5	67.5	22.1	32.7	1.0	n.e	n.e	n.e.	n.e	n.e	0.9
K. E., son	M	11	11.9	5.04	36.0	71.2	23.6	33.1	0.8	70.0	26.9	–	2.0	1.1	1.8
M. E., son	M	10	11.9	4.90	38.0	77.8	24.3	31.3	0.6	97.5	0		2.5	0	0.6

[1] Hb A_2 cannot be estimated in the presence of Hb G/S.
[2] Not examined.

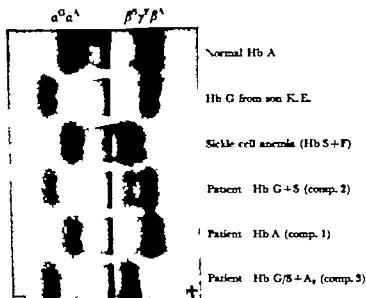


Fig 3. Starch gel electrophoresis in urea-veronal buffer pH 8.0 of the polypeptide chains of purified haemoglobin components.

soluble in the standard phosphate buffer solution. The haemolysates of the mother and the 2 other sons separated only into Hb A and Hb A_2, the Hb A_2 level being normal (2 4% in the mother and 2.5% in the eldest and youngest sons)

Polypeptide chain studies The different components were separated by column chromatography concentrated, dialyzed, and their purity was determined on starch gel electrophoresis The study of globins from the purified haemoglobin samples on starch gel electrophoresis in a urea veronal buffer at pH 8.0 revealed that the haemoglobin with the mobility of Hb A in the propositus and his 2 daughters indeed had normal α and β-chains. The component with the mobility of Hb S however had 4 polypeptide chains normal α- and β-chains and abnormal α and β-chains (fig 3) The abnormal β-chains resembled those of Hb S These findings indicate that the component with the mobility of Hb S actually consisted of 2 different haemoglobins (1) Hb S (whose presence explained the positive sickling) and (2) a haemoglobin whose mobility was similar to that of Hb S on starch gel electrophoresis at pH 8.6 but which had abnormal α-chains. The patient's haemoglobin component with the mobility of Hb A_2 showed a similar abnormal α-chain and an abnormal β-chain resembling that

Table II Amino acid composition of the 9a and 9b peptides of the second son K.E.

Amino acid	Peptide 9 Molar ratio Present work	Peptide 9 Molar ratio Literature value for Hb G	Number[1] of residues reported for Hb G	Peptide 9b (= 5b) Molar ratio Present work	Peptide 9b (= 5b) Molar ratio Literature value for Hb G	No. of residues reported for Hb G
Lysine	1.82	1.91	2	0.99	0.91	1
Histidine				3.04	2.28	3
Aspartic acid	1.19	1.09	1	4.39	4.01	4
Threonine	0.94	0.87	1			
Serine				1.76	1.83	2
Proline				0.90	1.04	1
Alanine	1.80	2.15	2	5.13	4.92	5
Valine	1.14	0.96	1	2.09	2.03	2
Methionine				0.16	(1)	1
Leucine	1.00	1.00	1	3.00	3.00	3

[1] Baglioni and Ingram [3].

[2] The greatest portion of the methionine in our analysis was oxydized to methionine sulfoxyde which appears on the chromatogram as a peak before the aspartic acid peak [11].

Discussion

From the clinical and haematological findings for the patient and his two daughters, in all of whom the abnormal genes for Hb G and Hb S were present simultaneously, we concluded that interaction between the 2 abnormal genes does not lead to significant impairment of health. However, mild anaemia may be present, and their peripheral blood showed slight anisocytosis and poikilocytosis and a few target cells. In the case of simultaneous occurrence of Hb G and Hb S described by Pugh *et al.* [12] the patient, a child, had severe anaemia and clinical symptoms. However, the condition in that case was complicated by homozygosity for the Hb S gene, itself leading to severe anaemia. The child's mother, on the other hand, was doubly heterozygous for Hb G and Hb S and had only mild anaemia and slight changes in the peripheral blood.

Biochemically, our patient and his two daughters, all with the same abnormal haemoglobin pattern, again provide evidence that normal and abnormal α-chains and normal and abnormal β-chains are produced independently, as reported by other investigators. Identical peptide chains form symmetrical dimers, which combine at random and

lead to the formation of 4 major haemoglobins. As in the case of Pooh *et al* [19] 2 haemoglobins besides Hb G and Hb S are formed, Hb A with normal α and β-chains and Hb G/S with abnormal α- and β-chains. Figure 3 shows that in our patient's component 2, which consists of Hb G and Hb S the spots indicating α^G and β^A are more pronounced than the spots indicating α^A and β^S which means that the amount of Hb G is higher than Hb S This is in agreement with the observations of Pooh *et al* in the only other known family with Hb G and Hb S Using a different quantitative technique they showed in their patient's mother who carried the genes for Hb G as well as for Hb S that the amount of Hb G was higher than the amount of Hb S They postulated that the presence of some type of non-allelic genetic regulator might have impaired the normal α-chain production. The decreased availability of α_2^A subunits would then result in lower proportion of Hb A and Hb S Whether this is always the case in Hb G and Hb S combinations cannot be said since the family of Pooh *et al.* and the one described in this paper are the only ones available for study

Genetically the family we have described is of interest because the propositus, who had two abnormal genes, was married to a normal woman. Since 2 of his children inherited both his abnormal genes, one child inherited only one abnormal gene, and 2 inherited no abnormal haemoglobin, it can be concluded that the genes are not alleles. Hence, the 2 abnormal genes are either located in 2 different chromosomes that were inherited independently or are linked in one chromosome but one son received only one abnormal gene due to crossing over Unfortunately descriptions of such critical matings have been too few to permit a more accurate evaluation of the chromosomal relationship of the genes. In the family reported by Pooh *et al.*, in which the genes for Hb G and Hb S were segregating, the observed inheritance of Hb G and Hb S was complicated because the husband was a Hb S trait carrier and therefore not normal. One child from this mating was heterozygous for Hb G and homozygous for Hb S, another was normal, and the other children were not available for study

As already mentioned, the segregation of the genes for Hb G and Hb Cβ has been described more often than the combination of Hb Gα and Hb Sβ. Since the genes for Hb C and Hb S are alleles, the mode of inheritance for Hb C also applies for Hb S and information regarding the chromosomal relationship between the Hb Sβ and Hb Gα genes can also be derived from families in which Hb C and Hb G are

segregating. Unfortunately again in the publications about such combinations, critical matings for genetic study were rarely reported. In the family described by RAPER *et al.* [13] a man doubly heterozygous for Hb G and Hb C was married to a normal woman and they produced one doubly heterozygous child and one Hb C trait carrier. Matings less suitable for genetic information have been reported by others. In the family described by WEATHERALL *et al.* [20] a man with the Hb C trait married a woman heterozygous for Hb G. Of the 5 offspring of this mating who were examined, one was normal, one heterozygous for Hb G and 3 were doubly heterozygous for Hb G and Hb C. In the family reported by RIEDER and NAUGHTON [14] a man homozygous for Hb C married a woman heterozygous for Hb G and they produced one child who was doubly heterozygous for Hb G and Hb C. In this case the Hb G was Hb G Baltimore instead of Hb G Philadelphia. In the family with Hb G and Hb C described by ATWATER *et al.* [1] the husbands of the women reported were not examined. Another combination of an α-chain abnormality with a β-chain abnormality was reported by HALL-CRAGGS *et al.* [5] in a family in which the genes for Hb S and for Hb Sta II were segregating. On starch gel electrophoresis the Hb Sta II had the same mobility as Hb S and was shown to be an α-chain abnormality but the fingerprints revealed that it differed from Hb G. Three matings in this family were available for study. In one the husband and wife both doubly heterozygous for Hb S and Hb Sta II produced 2 children one heterozygous for Hb S and one for Hb Sta II. In the second mating, a Hb S trait carrier married a woman doubly heterozygous for Hb S and Hb Sta-II. Five children resulted from this marriage two were heterozygous for Hb S, one was homozygous for Hb S and one doubly heterozygous for Hb S and Hb Sta II while the fifth was homozygous for Hb S and heterozygous for Hb Sta II. In the third mating, a man doubly heterozygous for Hb S and Hb Sta II who was twice married to normal women, fathered 4 children two were Hb Sta II trait carriers, one was normal, and one was doubly heterozygous for Hb S and Hb Sta II.

In another family in which abnormal α-chain and abnormal β-chain haemoglobins were found together Hb S was combined with Hb Hopkins-2 [18] a fast moving haemoglobin that later was shown to be abnormal in the α-chains. In the second generation of this family a critical mating took place between a woman doubly heterozygous for Hb S and Hb Hopkins-2 and a normal man resulting in 7 children. Of 6 of them studied, one was doubly heterozygous for Hb S and

Hb Hopkins-2, 3 were heterozygous for Hb S and 2 for the fast moving Hb Hopkins-2.

From the findings so far reported and from the additional family described in this paper a close chromosomal linkage between the genes for Hb Gα and Hb Sβ is indeed unlikely

Acknowledgment

We wish to thank Mr Robert [illegible] of the Hooper Foundation for technical help in this study

Summary

A second family with Hb G Philadelphia (an α-chain abnormality) and Hb S is described, in which the propositus, a Negro man, was doubly heterozygous for Hb G Philadelphia and Hb S and had 4 major haemoglobins Hb G, Hb S, Hb A, and Hb G/S. His wife was normal. Two of his five children were doubly heterozygous for Hb G and Hb S and had the same haemoglobin pattern as the father; two others were normal, and another child inherited only the gene for Hb G Philadelphia. In this family the interaction between the genes for Hb G Philadelphia and Hb S led to mild anaemia and slight changes in the peripheral blood.

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Effect of Androgen Antagonist on Erythropoietic Action of Testosterone

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It has already been established that the prolonged administration of androgen results in an increase in erythropoiesis either in experimental animals or in patients with various types of aplastic anemia or with breast cancer [1–4] although the exact mechanism remains unknown. Some investigators have recently suggested that androgenic steroid exerts its stimulatory effect on erythropoiesis through increased production of erythropoietin in the kidney [5,6]. One of the authors has pointed out the fact that this steroid has an ability to enhance a response of the erythroid tissue to hypoxic stimuli [7]. It is well known that androgen has both anabolic and virilizing activity.

The experiments presented in this paper were carried out in order to clarify the following questions: Which activity is responsible for an increase in erythropoiesis? What relationship exists between erythropoiesis stimulating activity and these two main activities of androgen?

Materials and Methods

Experiment I. Female rats of Wistar strain weighing 120 g were used as experimental animals, and divided into the following 5 groups. Each rat of group 1 received an intramuscular injection of 12.5 mg of testosterone as mixture of enanthate and propionate in 0.1 ml of sesame oil (Enarmon depot®, Teikoku-zoki Co.). Each rat of group 2 was injected with 12.5 mg of methenolone acetate (Primobolan depot®, Schering AG) intramuscularly. Each rat of group 3 or group 4 received an intramuscular injection of 12.5 mg of testosterone or methenolone respectively, and thereafter 10.0 mg of antiandrogenic agent, cyproterone acetate (1,2α-methylene-6-chloro-Δ^{6}-pregnadiene-17α-ol-3,20-dione 17α-acetate) in

This compound was generously supplied by Dr. F. Neumann, Schering AG, Berlin.

0.2 ml of a benzyl-benzoate-castor oil mixture [1 : 5(v/v)] was injected intramuscularly for a week. Group 5 was served as control, which received an intramuscular injection of both 0.1 ml of sesame oil and 0.2 ml of mixture of benzyl-benzoate-castor oil for week.

Experiment 2. Female mice of ddY-strain weighing 20 to 25 g were made plethoric by two daily intraperitoneal injection with 0.6 ml of an 80 percent suspension of washed homologous erythrocytes. The plethoric mice were divided into 9 groups. Each mouse of group A, B, C, or group D, E, F was given the following experimental procedures 6 days after single intramuscular injection with 2.5 mg of testosterone or with both similar dose of testosterone and 10 mg of cyproterone acetate, respectively: intramuscular administration of p.3 Co-units of purified erythropoietin(Ep) to group A and D, exposure to hypoxia of ½ atmosphere for 12 h to group B and E, and intramuscular injections with 0.3 ml of normal saline to group C and F. This injection with testosterone or with both testosterone and cyproterone acetate was carried out on the day of the last infusion of erythrocyte suspension. Six days after the last infusion of erythrocyte suspension, each mouse of group G, H or I was given the above described experimental procedures except the injection with testosterone or with both testosterone and cyproterone acetate.

Experiment 3. Seventeen plethoric mice similar to those used in experiment 2 were divided into 4 groups consisting of 4 to 6 mice. Each mouse of group J, K, L or M was injected with 0.6 Co-units of Ep, both 0.6 Co-units of Ep and 10 mg or 5 mg of cyproterone acetate, 0.6 ml of normal saline, respectively.

Quantitative determination of erythropoiesis was done as follows: rats were injected with 0.5 μc of radioiron ($^{59}FeCl_3$) intravenously 24 h after the last injection of mixture of benzyl-benzoate-castor oil or cyproterone. Mice were similarly injected with 0.5 μc of radioiron intraperitoneally 56 h after the last injection of normal saline, Ep, hypoxic stimulation. The percentage of ^{59}Fe incorporation into red cell mass was determined from the radioactivity of the blood sample taken by cardiac puncture on 24th h in rats and 72nd h in mice after the injection of radioiron, assuming the total blood volume of test animal is 5.0% (in rat) or 7.0% (in plethoric mouse) of the body weight.

Bioassay for erythropoietin was carried out by MIRAND's method [8]. Plasma samples obtained from each animal of the same experimental group were pooled and stored at -20°C until bioassay for erythropoietin activity was done. Activity of the erythropoietin in the pooled plasma sample was expressed as percentage of ^{59}Fe utilized by red cell mass of bioassay mice in 24 h.

Results

1. Effect of androgen antagonist on ^{59}Fe incorporation into red cell in testosterone or anabolic hormone treated rats. As shown in table I, significant increase in both ^{59}Fe incorporation and reticulocyte counts was noted in rats injected either with testosterone (group 1) or with methenolone (group 2). The activity of erythropoietin in pooled plasma obtained from these animals was evidently elevated as compared with control rats of group 5. There was, however, no significant difference in these values between group 1 and 2. Cyproterone administration did not suppress the enhancing effect of testosterone or methenolone both on ^{59}Fe incorporation into red cell and on erythropoietin activity in

Table I Effect of androgen antagonist on erythropoiesis and plasma erythropoietin level of female rats treated with testosterone or anabolic hormone

Experimental group	No. of animals	% ^{59}F incorp. into RBC	Reticulocyte count, %	Plasma erythropoiet in level, %
Rats injected with Testosterone (group 1)	5	34.6±5.9	6.26±1.18	4.53±1.38
Methenolone (group 2)	5	31.6±4.5	5.06±0.29	2.21±0.81
Both Testosterone and Cyproterone (group 3)	5	29.6±4.0	5.02±1.44	3.18±1.09
Both Methenolone and Cyproterone (group 4)	5	28.5±5.4	4.30±1.56	2.71±1.02
Normal control (group 5)	8	23.6±4.8	3.5±1.30	0.62±0.21

Mean±S.D. of the mean.

Table II. Effect of androgen antagonist on erythropoietic synergism of testosterone with erythropoietin or hypoxia

Experimental group	No. of animals	% ^{59}F incorporation into RBC
Mice pretreated with testosterone		
Erythropoietin (group A)	5	16.20±3.15
Hypoxia (group B)	4	12.38±2.48
Saline (group C)	5	3.65±1.83
Mice pretreated with both testosterone and cyproterone		
Erythropoietin (group D)	5	13.49±2.18
Hypoxia (group E)	4	8.13±2.33
Saline (group F)	5	2.76±1.67
Normal mice		
Erythropoietin (group G)	5	9.50±2.24
Hypoxia (group H)	6	3.44±1.12
Saline (group I)	5	0.42±0.13

Mean±S.D of the mean.

Table III. Effect of androgen antagonist on an enhancing ^{59}F incorporation into RBC in plethoric mice induced by exogenous erythropoietin

Experimental group	No. of animals	Hematocrit %	% ^{59}F incorporation into RBC
0.6 Co-U of erythropoietin (group J)	4	63.2±3.7	18.20±2.21
Both 0.6 Co-U of erythropoietin and 10 mg of Cyproterone (group K)	4	59.2±4.4	19.00±2.28
Both 0.6 Co.-U of erythropoietin and 5 mg of Cyproterone (group L)	4	58.5±5.5	16.22±4.00
Saline (group M)	5	62.0±3.0	0.88±0.22

Mean±S.D. of the mean.

plasma. Similarly cyproterone exhibited no inhibitory effect on an erythropoietic action of methenolone, as seen in table I

2 Effect of androgen antagonist on erythropoietic synergism of testosterone with erythropoietin or hypoxia. The results obtained are shown in table II The combined effect of administration of testosterone with an erythropoietin injection (group A) or exposure to hypoxia (group B) on ^{59}Fe incorporation into red cell exceeded the value calculated from the sum of each stimulus (group C, G and H) From the results obtained in mice of group A and D or group B and E, it was evident that the treatment with cyproterone did not change the synergistic effect of testosterone with erythropoietin or hypoxia on erythropoiesis.

3 Study of the inhibitory effect of androgen antagonist on erythropoiesis The results obtained in experiment 3 are listed in table III Administration of 5.0 or 10.0 mg of cyproterone did not exert influence upon the enhancing ^{59}Fe incorporation into red cell induced by an exogenous erythropoietin given simultaneously

Comment

It is noteworthy that methenolone has the same erythropoietic activity as testosterone at an equal dose level, despite the fact that the virilizing effect was one fourth of that of testosterone. This finding indicates that an erythropoiesis stimulating activity of testosterone may not directly related to its virilizing effect. DUARTE *et al.* have also reported similar

results [9] Cyproterone acetate used in these experiments has no estrogenic effect but blocking activity of androgen receptor in target organ [10 11] The results obtained hereabout reveal that cyproterone has no inhibitory effect on erythropoiesis stimulating action of either testosterone or methenolone. An increase in erythropoiesis induced by testosterone should be prevented by this androgen antagonist, if an erythropoietic action of testosterone is related directly to its androgenic effect. There are some evidences indicating that increase in erythropoiesis by testosterone results from an elevation in endogenous erythropoietin activity Cyproterone did not affect the action of exogenous erythropoietin. Thus, it is indicated from the results presented here that the erythropoietic activity of testosterone is at least independent of its virilizing effect. Administration of androgen antagonist could not inhibit a synergistic effect of testosterone with hypoxia or erythropoietin on erythropoiesis. It is also possible to say that these synergism might not be based upon the virilizing effect of testosterone, although mechanism on these synergism remains to be clarified.

Summary

The mechanism of erythropoietic action of testosterone, especially its relation to virilizing effect was studied, and the following results were obtained (1) At an equal dose level methenolone has the same erythropoietic activity as that of testosterone. (2) Treatment with sufficient amount of cyproterone acetate failed to suppress an erythropoietic effect of testosterone, methenolone or exogenous erythropoietin. Cyproterone acetate did not prevent significant increase in erythropoietin level in plasma by injecting testosterone or methenolone. (3) Synergistic effect of testosterone with exogenous erythropoietin or hypoxia on radioiron utilization was not affected by cyproterone acetate. From these results it is suggested that the erythropoietic action of testosterone is independent of its virilizing effect.

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Maintenance Therapy of Pernicious Anaemia

Comparison of Cyanocobalamin, Hydroxocobalamin and a Cyanocobalamin-Zinc-Tannate Complex

A. Killander and I. Werner

The proper evaluation of different compounds for maintenance therapy in pernicious anaemia has always been difficult. It is well known that following interruption of treatment the time at which a relapse occurs may vary considerably in different patients [19, 15, 9, 4]. In a previous communication we reported the serum B_{12} levels in 45 patients after discontinuation of B_{12} therapy [12]. Twenty patients reached low levels (100 pg/ml and less) within the first year and a further 20 within the second year. One patient was still normal after 26 months whereas the remaining 4 cases did not show low values until 44 months or more after the specific treatment had been discontinued. The reasons for this large individual variation are incompletely known.

The earliest sign of B_{12} deficiency is a subnormal serum level of this vitamin [13, 10]. By taking serial blood samples different B_{12} compounds can be compared, using the patient as the control. The results of such a study are presented in this paper. The substances tested were cyanocobalamin (CN B_{12}), hydroxocobalamin (OH B_{12}) and cyanocobalamin-zinc tannate complex (Zn B_{12}). These abbreviations are used throughout the paper.

Material

The series consisted of 22 patients with untreated pernicious anaemia. In all cases positive diagnosis was indicated by the presence of the following criteria: macrocytic anaemia, megaloblastic bone marrow, histamine-fast achlorhydria, serum B_{12} concentration of 100 pg/ml and less and typical response to B_{12} therapy. In 17 cases all 3 B_{12} preparations

were studied consecutively at least once. The remaining 5 patients for different reasons were given only 2 of the compounds. All vitamin B_{12} compounds used contained 1 mg/ml. The following preparations of the above mentioned substances were used: Hepagon forte® (cyanocobalamin), Redisol®, Hepagon novum® (hydroxocobalamin), *Depinar*® (*cyano*-cobalamin-zinc-tannate)[1].

Methods

Routine haematological methods were used. The assay of vitamin B_{12} activity in serum and urine was performed microbiologically using *Euglena gracilis* strain [8]. In our laboratory values of 100 pg/ml or less are defined as being abnormally low and indicating B_{12} deficiency. Values between 100 and 150 pg/ml are regarded as borderline and those between 150 and 900 pg/ml as normal. Urine was collected for 24 hour periods, kept in refrigerator at +4°C for not more than 48 h. Frozen aliquots were stored at –20°C until assayed. In some cases ^{57}Co- or ^{58}Co-labelled vitamin were used in the excretion studies. The radioactivity was measured in 1,000 ml volumes in a scintillation counter. The rate of absorption of the different compounds from the site of injection was studied using radioactive vitamin and surface counting.

Each patient received an initial injection of 1 mg of either CN B_{12}, OH B_{12} or Zn B_{12}. Haemoglobin concentration, red cell count, reticulocyte count and serum B_{12} levels were estimated at frequent intervals following the initial injection until normal blood levels were seen. After this period haemoglobin or haematocrit determinations and serum B_{12} levels were performed monthly or in some cases every second month. The interval from the injection to the first low serum B_{12} value was taken as measure of the duration of the therapeutic effect of the substance tested. After the second consecutive low value a second injection of another type of B_{12} was given. The haemoglobin or haematocrit values as well as the serum B_{12} levels were followed monthly as before. A third injection again of different substance was given following the finding of two further consecutive subnormal values. In some cases the experiment was continued for up to 3 more injection-relapse cycles.

Results

All 3 preparations were studied in 17 patients. Five of these had CN B_{12}, 5 had OH B_{12} and 7 had Zn B_{12} as their first injection. A full haematological remission was obtained in all patients by this initial injection of 1 mg of vitamin B_{12}. No differences were found between the groups of patients with regard to the time and height of the reticulocyte peak or the rate of increase in the number of red cells. Haematological remission was also maintained during the following experiments and no neurological symptoms or signs developed in any patient. Nor were any other symptoms encountered which could be attributed to B_{12} deficiency.

[1] We are greatly indebted to AB Astra, Sweden, Merck, Sharp & Dohme Inc., Rahway, N.J., USA and Armour Pharmaceutical Company, Kankakee, Ill., USA for the gift of these preparations.

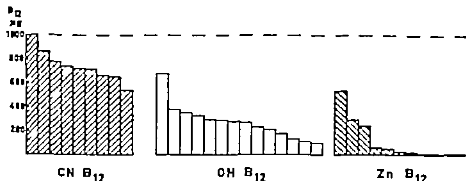


Fig 1 Seventy-two hour urinary excretion of vitamin B_{12} after single injection of 1 mg of CN B_{12}, OH B_{12} and Zn B_{12}. Each block represents 72 hour period in one patient.

Urinary Excretion

The 72 hour urinary excretion of CN B_{12}, OH B_{12} and Zn B_{12} was studied in 9 13 and 11 patients respectively Nine patients were subjected to 2 or 3 excretion studies. The excretion of CN B_{12} and OH B_{12} is negligible after 72 h. In the case of Zn B_{12} the situation is variable. Usually there is a protracted excretion between 72 h and 168 h, in some cases amounting to 5–10%. The results are presented in figure 1 The mean and range of excretion for the 3 substances were CN B_{12} 746 μg (543–1 000) OH B_{12} 284 μg (99–647) and Zn B_{12} 112 μg (3–535) Although there was considerable variation between the different patients it was evident that much more was lost after a single injection of 1 mg of CN B_{12} than of the other 2 compounds tested. About 75% of the CN B_{12}, 30% of the OH-compound and 10% of the Zn B_{12} was lost in the urine during the first 3 days.

In a few cases excretion studies were performed using both an isotope technique and microbiological assay In the case of CN B_{12} alone or in the zinc-tannate complex there was good agreement between the results obtained from the two methods. In some cases that were given OH B_{12} the excretion of radioactivity was considerably higher than the corresponding microbiological activity The reason for this discrepancy was most probably that the urine had been left at room temperature We have found that urine samples left at room temperature will at times cause a fall in B_{12} activity when OH B_{12} is present but not with solutions of CN B_{12}. The figures given for OH B_{12} excretion thus

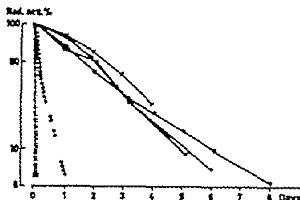


Fig. 2. Rate of absorption of 1 mg radioactive CN B_{12} (———) OH B_{12} ( ) and Zn B_{12} (——) from the site of injection.

represent minimum values as it cannot be excluded that some of the urine samples may have been subjected to mishandling as mentioned above

Rate of Absorption

This was studied in 7 patients. A possibility of a difference between subcutaneous and intramuscular administration was investigated in one patient. One mg of radioactive CN B_{12} was injected intramuscularly in the gluteal region and subcutaneously in the thigh on 2 consecutive days There was a slight difference in the absorption rate between the 2 routes of administration Fifty % of the dose had disappeared 42 min after the intramuscular injection and 48 min after the subcutaneous injection. In the other 6 patients the radioactive compounds were only given intramuscularly The absorption rate of the 3 compounds differed considerably as illustrated in figure 2 Thus, 50% of the injected dose of CN B_{12} disappeared 26 and 42 min after the injection (2 cases) The corresponding figures for OH B_{12} was 2 5 h (one case) and for Zn B_{12} 1 6 to 2 4 days (4 cases)

The Serum B_{12} Levels

The time from the injection to the first serum B_{12} level found to be 100 pg/ml and below was considered as the duration of the therapeutic

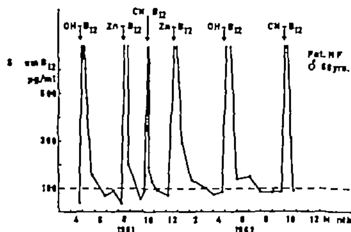


Fig. 3. The serum B_{12} values of case H.F

effect. This level was chosen since all patients, when started on therapy had serum B_{12} values below this level. In order to ascertain that a stable subnormal B_{12} level had been reached the next injection was not given until a second low value had been obtained. In this way the conditions at the time of injection were considered to be as standardiz ed as possible without exposing the patients to the risks of a relapse. It is reasonable to believe that the B_{12} deficiency was most pronounced at the time of the first injection. For this reason the 3 compounds were injected in random order In this way other possible systematic errors were also avoided

The results showed a considerable individual variation, the extremes are represented by patient H. F and K. J Their serum B_{12} levels after the injection of the different compounds are illustrated in figure 3 and 4 In the case of H F the effect of the first CN B_{12} injection did not even last one month. The effect of the long-acting preparations was 2 to 3 months. In the case of K. J the effect of CN B_{12} lasted 2.5 months. After the injection of Zn B_{12} the serum B_{12} level remained above 100 pg/ml for 18 months. After the same amount of OH B_{12} the B_{12} levels were above 100 pg/ml for more than one year when the patient died from a cerebral haemorrhage.

The data from all 22 patients are shown in figure 5 The mean values are given in table I It is evident that the duration of the therapeutic effect of OH B_{12} and Zn B_{12} is considerably longer than

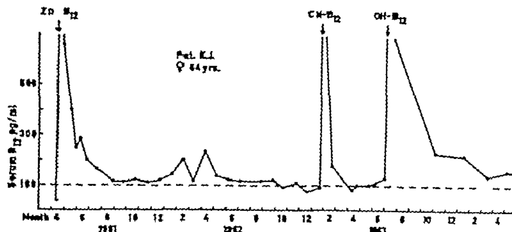


Fig 4. The serum B_{12} values of case K.J.

CN B_{12} OH B_{12} Zn B_{12}

Fig 5. The duration of serum B_{12} levels above 100 pg per ml following injections of 1 mg of CN B_{12}, OH B_{12} and Zn B_{12}. The preparations are pictured in the order of administration.

Table I

Substance	No. of injection-relapse-cycles	Duration of cycle in months	
		mean	range
Cyanocobalamin	21	2.1	1–7
Hydroxocobalamin	20	7.1	2–22
Cyanocobalamin-zinc-tannate	22	6.3	2–18

that of CN B_{12}. The results also suggest that the effect of CN B_{12} given as the first injection was much shorter (1 to 2 months) than the effect of the same compound given as second or third injection (1 to 7 months). No significant difference related to the order of injections was found between the other two preparations. The explanation for this prolonged effect of cyanocobalamin was that there probably remained a certain depot effect of the previously given OH B_{12} or Zn B_{12}. A depot effect of this kind might well influence the short period of normal B_{12} level after injections of CN B_{12} but be of no significance in the longer periods following injections of OH B_{12} or Zn B_{12}. However the number of the observations was too small to permit any definite conclusions. No significant differences could be observed between the duration of the effects of OH B_{12} and Zn B_{12}.

Discussion

During the last few years several reports on the therapeutic effect of so-called long acting B_{12}-preparations have been published. The preparation studied are in principle of two types. One is the OH B_{12}, the protracted effect of which is due to its affinity to both specific and unspecific binding proteins in blood and tissues [7]. The other type is represented by preparations in which either zinc-tannate [18] or tannate in a sesame oil/aluminium monostearate gel [16] have been added to CN B_{12} in order to decrease its solubility. In this study only the zinc-tannate-cyanocobalamin complex was used. The cyanocobalamin tannate complex in aluminium monostearate/sesame oil gel (B_{12} TAM®-Dumex) was not available when this investigation was started.

Several reports have been published comparing the effect of OH B_{12} with that of CN B_{12}. It is evident that the urinary excretion of OH B_{12} is much lower than that of CN B_{12} [5 11 6] The large individual variation found by us has been reported by several authors and there are also considerable differences in the excretion data reported by different laboratories.

The absorption rate from the injection site was studied by GLASS *et al.* [5] and by KILLANDER and SCHILLING [11] They found a slower rate of absorption with OH B_{12} compared to CN B_{12}. Our results agree with their findings. In our 4 cases that were given Zn B_{12}, 50% of the radioactivity had disappeared by about 2 days. No other reports on the absorption rate of Zn B_{12} seem to have been published. KRISTENSEN *et al* [14] working with B_{12} TAM found an appreciable activity at the site of injection 2 months after the administration of 1 mg The absorption rate measured as the disappearance of 50 % of the radioactivity was about 20 days. The absorption rate for the TAM preparation thus seems to be extremely slow compared to that of the substances studied in the present investigation.

Serum B_{12} levels after single injections of OH B_{12} have been reported by several authors. They all found much higher levels both initially and after several weeks when compared with CN B_{12} in pernicious anaemia patients [2, 20, 3] After a single injection of 1 *mg* of OH B_{12} normal values of serum B_{12} were observed in several patients for periods of 6 to 13 months. The present study clearly demonstrates these differences between the hydroxo- and cyanoforms of the vitamin, although the individual variation is considerable (fig 5) No other reports are available of the effect of Zn B_{12} on the serum B_{12} levels in pernicious anaemia patients. Our results indicate an effect quite similar to that of OH B_{12}. Studies on the B_{12} TAM have also shown a protracted effect of the same order of magnitude [1]

It is evident that the urinary excretion of vitamin B_{12} is much less after the injection of OH B_{12} and Zn B_{12} than after CN B_{12}. Both OH B_{12} and Zn B_{12} are able to maintain a normal serum B_{12} level for a much longer time than CN B_{12}. These two points indicate that better results can be obtained with these 2 preparations than with CN B_{12}.

The close similarity of the effects of OH B_{12} and Zn B_{12} is somewhat surprising To judge from the low disappearance rate and the urinary excretion figures a much longer duration of the zinc-tannate compound would have been anticipated. A prolonged urinary excretion of Zn B_{12} observed in a few patients might be one explanation. A defect utiliza

tion of the compound would also explain the above findings. The OH B_{12} represents a more physiological form of the vitamin and it can be assumed that what is retained is also used and metabolized normally in the body Zn B_{12} is an artificial, non-physiological compound of the vitamin and zinc tannate. Without further evidence it cannot be assumed that this highly insoluble material will be completely utilized by the body

Summary

A comparative study was performed on 3 types of vitamin B_{12} cyanocobalamin (CN B_{12}) hydroxocobalamin (OH B_{12}) and cyanocobalamin-zinc-tannate complex (Zn B_{12}) The rate of absorption from the site of injection of CN B_{12}, OH B_{12} and Zn B_{12} measured as the disappearance of 50% of the radioactivity was approximately 40 min, 2.5 h and 2 days respectively The 72-hour urinary excretion rate after injection of 1 mg of the B_{12} preparation was on the average 75% for CN B_{12}, 30% for OH B_{12} and 10% for Zn B_{12}. A complete haematological remission was obtained in all cases after single injection of 1 mg of all three substances. The serum B_{12} level was maintained bove 100 pg/ml for an average time of 2.1 months with CN B_{12}, 7 1 months with OH B_{12} and 6.3 months with Zn B_{12}. It is concluded that the effect of OH B_{12} and Zn B_{12} is superior to that of CN B_{12}.

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DNA Synthesis by Erythroblasts in Normal Man and in Some Disorders of Erythropoesis

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At present H-thymidine, a specific precursor of DNA, is generally used for the study of the proliferation of the bone marrow cells. Our paper presents investigations on the proliferative activity of the erythroblasts in 13 normal subjects and in 20 patients with marked disorders of erythropoesis.

Materials and Methods

Bone marrow samples were obtained by sternum puncture and were cultivated in liquid medium containing the patient own plasma and Hanck isotonic solution with heparin. ^{3}H-thymidine (specific activity 3.6 c/g) was added to the medium in the end concentration equal to 0.1 μc/ml. The bone marrow suspension in the nutrient medium was carried in 4 tubes in 1 ml and cultivated in shaking incubator at 37° C. I the first tube the incubation was stopped after 15 min, in the other tubes after ½, 1 and 2 h. After the incubation the tubes were centrifugated and smears were prepared from the sediment and fixed with methanol during 10 min. Autoradiography was carried out by the method of ZABOROV [21] A liquid nuclear emulsion of the R-type was used

The smears were stained using BOYD's technique [5]. Thirty to 40 young cells (pronormoblasts and basophilic normoblasts) 100 polychromatophilic normoblasts and 50 orthochromic normoblasts were calculated. The percentage of labeled cells was calculated in each group of cells.

The generation time of cells was calculated using the formula of DOMBUA and DOMBUA [8] for the exponentially growing population

$$T = \frac{\ln^{2}}{} \quad \text{where} \quad = \frac{\Delta N}{(N + N_p)\,\Delta t}$$

T = generation time. ln = 0.693. ΔN = accretion of the number of cells during time t. N = number of cells in period of DNA synthesis. N = proliferative pool, i.e. the ration of cells capable for division and all cells of the given species.

Emulsion R is produced at the Research Chemo-photographic Institute, Moscow (USSR)

Table 1 Clinical and hematological data of 15 patients with hypoplastic and iron deficiency anemia

Pat. No.	Diagnosis	Age years	Sex	Length of disease, years	RBC/mm³ × 10⁶	Hb, g%	FI	WBC/mm³ × 10³	Leucocytes %: staff cells	Leucocytes %: segmented neutrophils	Leucocytes %: lymphocytes	Sedimentation rate mm/h	Reticulocytes, %	Thrombocytes/mm³ × 10³	Serum iron, mg%
1	Hypoplastic anemia	48	F	0.3	3.1	11 7	1 1	5.3	2.5	38	59	11	1.8	95	
2	Hypoplastic anemia	63	M	0.3	3.0	10.6	1.0	4.6	14.5	20	37.5	10	1.2	20	
3	Hypoplastic anemia	27	F	0.5	1 4	4.33	0.92	2.0	22	17	53	8	–	10	
4	Hypoplastic anemia	19	F	1.2	2.5	6.33	1.0	2.0	10	29	49	20		10	
5	Hypoplastic anemia	14	M	0.2	3.0	11.33	1 1	3.26	1	8	82	20		37.5	
6	Chronic posthemorrhagic anemia	29	F	0.73	4 46	9.8	0.67	5.5	0.5	72.5	15	16	2.3	3.79	[illegible]
7	Chronic posthemorrhagic anemia	31	M	5	3.9	9.5	0.73	4.7	2	63	25	–			
8	Chronic posthemorrhagic anemia	47	F	1	3.3	9.5	0.81	3.5	1.5	51	32	22		318	[illegible]
9	Chronic posthemorrhagic anemia	48	F	1	4.8	8.2	0.5	5.5	1	74	11.3	11	0.8	328	
10	Chronic posthemorrhagic anemia	40	F	0.8	4.0	9.2	0.68	6.33	6	63.5	22.5	11	1.2		50
11	Chronic posthemorrhagic anemia	50	F	2	4.4	9.8	0.63	4.6	0.5	69.5	21.5	17			55
12	Chronic posthemorrhagic anemia	34	M	3	5.2	10.3	0.6	5.1	2.5	55.5	32.5	12	1	296	40
13	Essential hypochromic anemia	37	F	3	4.7	10	0.8	5.1	1	59	34	12	0.7	220	20
14	Essential hypochromic anemia	41	F	0.5	4 4	9.4	0.73	4.7	0.5	60.3	30.5	12		240	50
15	Essential hypochromic anemia	46	F	16	3.6	8.5	0.7	4.7	0.5	70	21.5	12	0.2		80

Table II. Clinical and hematological data of patients suffering from polycythaemia vera

Pat. No.	Age years	Sex	Length of disease years	RBC/ mm³ ×10⁶	Hb, g%	WBC/ mm³ ×10³	Thrombocytes/mm³ ×10³	Sedim. rate mm/h	Viscosity of blood	Hematocrit %	Increase of liver	Increase of spleen
1	39	M	1	6.44	21.0	13.0	520	2	16.6	83/17	+	+
2	48	M	14	8.1	18.3	7.2	130	1.5	8.5	70/30	+	+
3	42	F	2	6.08	17.6	14.2	600	2	10	80/20	+	+
4	58	M	3	6.95	21.0	29.2	400	2	12.5	79/21	+	+
5	34	F	5	7.25	23.6	4 4	170	1	16.6	88/12	+	+

It was assumed in the calculations that N t constant concentration of ³H-thymidine is equal to 1 i.e. all cells after period of DNA synthesis are divided and their destruction is minimal.

Control subjects were donors selected for bone marrow transplantations. Among the patients 5 had hypoplastic anemia 5 polycythaemia vera and 10 iron-deficiency anemia (7 chronic posthemorrhagic and 3 essential hypochromic anemias). The clinical and hematological data of the patients are shown in tables I and II.

Results

1 Normal subjects After 15 min incubation of bone marrow in the presence of ³H-thymidine the percentage of the label in pronormoblasts and basophilic normoblasts was 76.6 ± 0 73 and in polychromatophylic normoblasts 23 0 ± 0 86. After 2 h incubation there was a significant increase of the label ($p < 0.01$) up to 88.8 ± 1 1 and 37.6 ± 2.4 correspondingly (orthochromic normoblasts did not incorporate ³H thymidine)

The generation time of pronormoblasts and basophilic normoblasts was 17.8 ± 0.94 h, and that of polychromatophilic normoblasts 11.2 ± 1 1 h.

2. *Hypoplastic anemia.* After 15 min of cultivation the percentage of the label was 31.5 ± 6.06 in pronormoblasts and basophilic normoblasts and 8.2 ± 2.04 in polychromatophilic normoblasts. After 2 h the percentage increased up to 66.5 ± 5 03 and 14 6 ± 1.34 correspondingly

The generation time of pronormoblasts and basophilic normoblasts was 4.9 ± 0 6 and that of polychromatophilic normoblasts 17 6 ± 3.72 h.

3 Polycythaemia vera After 15 min cultivation the percentage of the label was 68.2 ± 1.87 in pronormoblasts and basophilic normoblasts and 19 7 ± 2 45 in polychromatophilic normoblasts. After 2 h it was 85.5 ± 2.93 and 33 3 ± 4 1 correspondingly

The generation time of pronormoblasts and basophilic normoblasts was 11 7 ± 1.2 h, and in polychromatophilic normoblasts it was 11 6 ± 0.9 h.

4 Chronic posthemorrhagic anemia. After 15 min cultivation the percentage of the label was 69 8 ± 3 66 in pronormoblasts and basophilic normoblasts and 24.3 ± 3 65 in polychromatophilic normoblasts. After 2 h incubation the label was 86.9 ± 1 9 and 32.5 ± 2.6 correspondingly

The generation time of pronormoblasts and basophilic normoblasts was 12.7 ± 1.5 h and that of polychromatophilic normoblasts 17.5 ± 3 39

5 Essential hypochromic anemia. After 15 min incubation the percentage of the label was 76.3 ± 2 0 in pronormoblasts and basophilic normoblasts and 22.0 ± 0.26 in polychromatophilic normoblasts. After 2 h it was 87 6 ± 3.9 and 31 6 ± 1.2 correspondingly

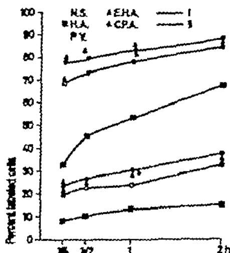


Fig 1 Uptake of ³H-thymidine in erythrocytic precursors of healthy subjects and patients with some disorders of erythropoesis. ● normal subjects, ■ hypoplastic anemia, ○ polycythaemia vera, △ essential hypochromic anemia, ▲ chronic posthemorrhagic anemia. —— pronormoblasts and basophilic normoblasts. – polychromatophilic normoblasts.

The generation time of pronormoblasts and basophilic normoblasts was 22.7 ± 5.83 h and in polychromatophilic normoblasts 16.0 ± 4 16 h.

All results are demonstrated graphically in figure 1

Discussion

The results of the present study in normal subjects are in agreement with those of other authors [1–3 6 7 11 13 16 19 20]

There are only few publications on the DNA synthesis of bone marrow cells in hypoplastic anemia [1 9 10 12, 14 15 17] According to these results our studies indicate a decrease of the cells synthesizing DNA and of the intensity of DNA synthesis. The marked fall of labeled cells after 15 min incubation also illustrates the decrease of DNA producing cells in the bone marrow of patients with hypoplastic anemia. The observation during 2 h shows that in these patients the increase of the label of pronormoblasts and basophilic normoblasts is 3 times greater than in normal subjects. However the label does not reach the normal values by the end of incubation. The high increase of the labeled pronormoblasts and basophilic normoblasts can be explained by a shortened presynthetic period G in the mitotic cycle of these cells, that is followed by an increased proliferative possibility of a small part of pronormoblasts preserving their ability for DNA synthesis. A marked shortening of the generation time confirms this assumption.

In hypoplastic anemia the slight increase of labeled polychromatophilic normoblasts and the more prolonged generation time may signify a prolongation of the presynthetic G period and, possibly of DNA synthesis.

The significant decrease of the label ($p < 0.01$) after 15 and 30 min incubation of the bone marrow of patients with polycythaemia vera confirms a diminution of the cells taking part in proliferation. It is evident that a considerable part of cells is in the 'rest period (G_0-period) The appearance of substances like inhibitors [8] or chalones [4] in the bone marrow of these patients cannot be excluded. These substances are contained in mature erythrocytes and prevent an entry of young cells into the mitotic cycle. By the end of the incubation the percentage of the label is almost normalized, that indicates the accelerated passing of cells through all periods of the mitotic cycle. This is

confirmed by a shortening of the generation time of pronormoblasts and basophilic normoblasts. The shortening of the mitotic cycle of pronormoblasts and basophilic normoblasts may be one of the factors that lead to the pathological increase of erythrocytes in polycythaemia vera.

There is no significant difference in the percentage of labeled cells between normal subjects and patients with iron deficiency. Thus there is no disturbance of DNA synthesis of the erythroid cells of patients with iron deficiency anemias. At the same time the generation time of polychromatophilic normoblasts is increased.

It is evident that the latter depends on the delay of polychromatophilic normoblasts in the presynthetic period (G_1) that may be stipulated by the disturbance in hemoglobinisations. It is of interest, that the generation time of pronormoblasts and basophilic normoblasts in essential hypochromic anemia was 10 h longer than in chronic posthemorrhagic anemia.

Summary

The DNA synthesis of the erythroid cells was investigated in bone marrow cultures by an autoradiographic method using ^{3}H-thymidine. A decrease of the label was found in hypoplastic anemia indicating a considerable diminution of the proliferative pool of erythroid cells. There was slight decrease of labeled cells in polycythaemia vera at the beginning of incubation indicating some diminution of proliferation. However by the end of incubation period the percentage of the labeled cells was normalized. In iron deficiency anemias the ^{3}H-thymidine incorporation did not differ from that in normal subjects.

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Change from *Fetal to Adult* Hemoglobin in Relation *to* Changes in the A Antigen of Human Erythrocytes

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Fetal erythrocytes differ from those produced in adult individuals not only in size and in the type of hemoglobin but also in antigenic properties of the cell membrane. Fetal erythrocytes possess an antigen that is no longer present in red cells of adults [6] and the ABH antigens are weaker in fetal red cells than in those of adults. Strength or reactivity of the A antigen increases during fetal life and infancy until adult values are reached at 2 to 3 years of age, thereafter remaining relatively unchanged for the rest of the individual's life [2] Change of the A antigen therefore overlaps the change from fetal to adult hemoglobin that takes place during the last trimester of gestation and early infancy At birth both the A antigen and hemoglobin are in process of undergoing change.

The question arose as to whether change in the A antigen and change in hemoglobin are closely interrelated. This was determined by utilizing blood samples of newborn infants and multivariate analyses. Results on the interrelation between strength of the A antigen and percentage of adult hemoglobin, as well as findings on effects of the infant's sex and birth weight are presented in this report.

Materials and Methods

Freshly collected samples of blood from the umbilical cord were used. Strength of the A antigen and blood group subtypes of A were determined as previously described [3, 4].

A modification of the technique of SINGER *et al.* [8] was used for determining fetal hemoglobin. KOH solution was used at 1/10 N, and the 1 N solution was stored at 4°C. The $(NH_4)_2SO_4$ solution was saturated at 25 C, diluted to 73% saturation, and also stored at

4 C. Washed and packed red cells were hemolyzed with distilled water and toluene. After centrifugation, the 2 upper layers were discarded and the red solution filtered through double Whatman No. 2 paper filter. With the filtrat and distilled water hemoglobin solutions were prepared that gave reading of exactly 500 units in Klett-Summerson colorimeter with green filter (corresponding to O.D. = 1.00). Three test tubes (size 13 x 100 mm) each with 1.5 ml of the standardized hemoglobin solution, were prepared from each sample of blood. Temperature was standardized to 25° C in constant temperature water bath. T each tube 1.0 ml of 1/10 N KOH, which had been prewarmed to 25 C, was added at determined intervals. After exactly 5 min of incubation, 2.0 ml of cold $(NH_4)_2SO$ solution was added to each tube. The tubes were inverted twice and the content filtered through dry No. 2 Whatman paper filter. Filtration was repeated through the same filter at 15 min after the addition of $(NH_4)_2SO_4$. Optical density was determined twice: first, after the second filtration t 30 min after the addition of $(NH_4)_2SO$ and again, after the third filtration at 45 min after the addition of $(NH_4)_2SO_4$. Percentage of fetal hemoglobin of sample was computed from the mean of the 6 readings (3 tubes measured twice) and the values converted to adult hemoglobin (100 minus percentage of fetal hemoglobin = adult hemoglobin).

Correlation coefficients were used, as more meaningful presentation of the relative magnitude of association between variables is possible than with regression coefficients. Partial correlation coefficients, form of multivariate analyses, have been described, for example, by KENDALL and STUART [7].

Percentage of fetal hemoglobin was determined in cord blood samples of 97 infants. *Only 86 infants, however could be included in the analysis as 6 mothers were uncertain* about their estimated date of conception; 5 non-Caucasians were not included because of known racial differences in birth weight and development [5]. The strength of the A antigen was determined in 54 A_1 Caucasian infants born to mothers of blood type A. Two girls, one resulting from 250 and an other from 297 day pregnancy represented the extremes in duration of pregnancy among the infants in whom strength of the A antigen had been determined. Duration of gestation ranged from 210 days (boy) to 305 (girl) among the infants in whom fetal hemoglobin had been determined.

Results

Figure 1 shows the frequency distribution for percentage of adult hemoglobin in the 86 infants and strength of the A antigen in the 54 A_1 infants. The figure reveals wide variation between infants. By coincidence the values for percentage of adult hemoglobin and strength of the A antigen are of similar magnitude.

In 24 infants, both strength of the A antigen and percentage of adult hemoglobin were determined. The results, illustrated in figure 2 reveal a positive association between the two variables. The simple correlation coefficient between strength of the A antigen and percent age of adult hemoglobin was $r = 0.52 \pm 0.12$ which, for 22 DF is highly significant ($P < 0.01$). Part of this association might result from a common effect of duration of gestation or a common effect of weight. These effects were corrected with partial correlation analyses.

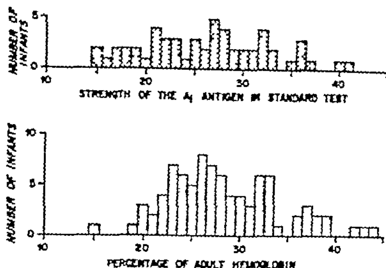


Fig. 1 Frequency distribution of strength of the A antigen and percentage of adult hemoglobin in newborn infants.

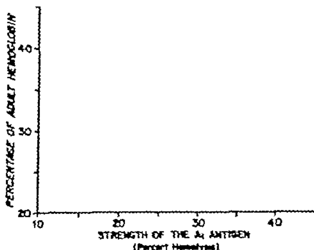


Fig. 2. Association in infants in whom both strength of the A antigen and percentage of adult hemoglobin were determined. The dots represent individual values.

The partial correlation coefficient (holding both birth weight and duration of gestation constant) was $r_{AH \cdot WG} = 0.44 \pm 0.18$ for 20 DF thus is significant ($P < 0.05$). Thus, there remains a significant association between strength of the A antigen and percentage of adult

Table I. Mean percentage of adult hemoglobin and mean strength of the A antigen in newborn infants

Sex	Number of infants	Mean duration of gestation days	Mean birth weight kg	Mean percentage of adult hemoglobin
Boys	50	278.4 ± 1.9	3.36 ± 0.35	27.49 ± 0.80
Girls	36	283.8 ± 1.7	3.34 ± 0.43	29.54 ± 1.00
				Mean strength of the A antigen
Boys	31	280.9 ± 1.3	3.[illegible]0 ± 0.48	24.78 ± 1.11
Girls	23	278.5 ± 2.4	3.44 ± 0.44	28.62 ± 1.52

Strength determined in immunohemolytic test

hemoglobin that is independent of birth weight and of duration of gestation.

Means and standard errors for percentage of adult hemoglobin and strength of the A antigen are presented in table I for boys and girls separately. Percentage of adult hemoglobin was slightly higher in girls than in boys but the difference was not statistically significant ($P > 0.05$). Duration of gestation was significantly lower in boys than in girls and this accounts in part for the observed higher mean percentage of adult hemoglobin in girls than in boys. A slight difference, however was still discernible among the infants resulting only from pregnancies of 270 to 290 days duration: the mean percentage of adult hemoglobin for girls was 29.7 ± 1.23 and that for boys was 28.0 ± 0.96.

Strength of the A antigen, on the other hand, was significantly higher in girls than in boys ($P < 0.05$). As table I shows, the average duration of gestation was slightly higher for the 31 boys than for the 23 girls, i.e., an even larger difference in antigen strength may be expected for equal duration of gestation.

Simple and partial correlation coefficients are presented in table II. The table shows that the simple correlation coefficient between percentage of adult hemoglobin and duration of gestation was slightly higher than that between percentage of adult hemoglobin and birth weight. Both were highly significant ($P < 0.01$). The partial correlation coefficients, holding either duration of gestation or birth weight constant, were markedly lower than the corresponding simple correlation coefficients.

Table II. Simple and partial correlation coefficients of percentage of adult hemoglobin and strength of the A antigen with birth weight and duration of gestation

		Birth weight (W)	Duration of gestation (G)
Percentage of adult hemoglobin (H)	Simple corr coeff.	= 0.34[2] ± 0.10	r = 0.56[2] ± 0.09
	Partial corr coeff.	$r_{HW.G}$ = 0.18 ± 0.11 (holding duration of gestation constant)	$r_{HG.W}$ = 0.24[1] ± 0.10 (holding birth weight constant)
Strength of the A antigen (A)	Simple corr coeff.	r = 0.42[2] ± 0.11	r = 0.21 ± 0.13
	Partial corr coeff.	$r_{AW.G}$ = 0.31 ± 0.13 (holding duration of gestation constant)	$r_{AG.W}$ = 0.03 ± 0.14 (holding birth weight constant)

[1] P < 0.05 [2] P < 0.01

Table II further reveals that strength of the A antigen is more closely correlated with birth weight than with duration of gestation, the partial correlation coefficient between strength of the A antigen and duration of gestation being close to zero. The partial correlation coefficients thus show a trend in the opposite direction: strength of the A antigen was closer correlated with birth weight than with duration of gestation, while percentage of adult hemoglobin showed a slightly closer association with duration of gestation than with birth weight.

Discussion

Newborn infants revealed wide variation in strength of the A antigen of red cells and in percentage of adult hemoglobin. The analyses disclosed that part of this variation resulted from variation in duration of gestation and variation in birth weight. However, holding duration of gestation and birth weight constant in partial correlation analysis, still revealed a significant association between strength of the A antigen and percentage of adult hemoglobin. An association between strength

of the A antigen and percentage of adult hemoglobin was thus demonstrated that is independent of variation in duration of gestation and variation in birth weight. Despite this association, the results have also provided evidence that change in the A antigen and change in hemoglobin are not fully interdependent. This was demonstrated by the following findings (a) the correlation between strength of the A antigen and *percentage of adult hemoglobin was less close than could* be expected if the change were fully synchronized (fig 2) (b) strength of the A antigen was much closer correlated with birth weight than with duration of gestation, while percentage of adult hemoglobin was slightly closer correlated with duration of gestation than with birth weight (c) an apparent effect of sex was more marked for strength of the A antigen than for percentage of adult hemoglobin (d) change in hemoglobin is completed at an age before strength of the A antigen has reached adult values [2] These findings suggest that change in the A antigen and change in hemoglobin may be controlled by different factors.

The partial correlation coefficients determined in the present study may be used to provide a measure of the causes that affect the change in hemoglobin and the change in the A antigen, in particular to determine whether change is predominantly a function of chronological age or of the infant's development (expressed in birth weight) Either of the two might be feasible. Duration of gestation, however was found to be an almost negligible determinant for the A antigen when compared with the effects associated with birth weight. The significant partial correlation coefficient between strength of the A antigen and birth weight (holding duration of gestation constant) suggests that relative development of the infant (expressed in weight) has a marked effect on the change in the A antigen. In general, rapidly developing infants (expressed in birth weight) are expected to show a relatively faster increase in strength of the A antigen than slower developing infants. This conclusion is further supported by the finding of a higher strength of the A antigen in girls than in boys. Girls are known to develop faster than boys (physiologic, skeletal) despite the fact that boys exceed girls in size and weight [1 9] In connection with properties of the erythropoietic system, the faster skeletal development in girls than in boys, as reported by Citarrie [1] is of particular interest.

In contrast to the A antigen, percentage of adult hemoglobin was found to occupy more an intermediate position in its association with *duration of gestation and birth weight. The significant partial corre*lation coefficient between duration of gestation and percentage of adtul

hemoglobin indicates that the change from fetal to adult hemoglobin is markedly influenced by chronological age of the infant. Thus, change in hemoglobin seems to be more dependent on time than on the infant's weight, while for the A antigen the reverse is the case.

The significant partial correlation coefficient between strength of the A antigen and birth weight indicates also that weight is not merely an expression of size, but may provide information on the relative development of the infant. This has long been known to be valid in cases of prematurity. The present results reveal, however, that this is applicable even for infants resulting from pregnancies of the same duration.

Summary

Strength of the red cell A antigen and level of adult hemoglobin were found to be significantly correlated in newborn infants, even after correction for variation in birth weight and duration of gestation. Furthermore, strength of the A antigen was significantly higher in girls than in boys and much closer associated with birth weight than with duration of gestation. On the other hand, percentage of adult hemoglobin was somewhat closer associated with duration of gestation than with birth weight. The results indicated that relative development of the infant (expressed in birth weight) has a marked effect on the change in the A antigen, while the change in hemoglobin is more dependent on chronological age of the infant.

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Congenital Deficiency of Fibrin Stabilizing Factor (Factor XIII)

A Report of the First Case from Latin America

T. Arends, J. M. Guevara and J. R. Rangel

The cases of congenital deficiency of the fibrin stabilizing factor (FSF) (synonyms: factor XIII, Laki and Lorand factor, fibrinase, Atoka diathesis) described to date have been found in Europe, Switzerland [1], Finland [2, 3], France [4, 5], Italy [6, 7], England [8], Greece [9] and Denmark [10]. It has also been reported in Israel [11], in the United States [12–13] and Canada [14]. No cases have been previously reported in Latin America.

The case reported here concerns a FSF deficiency studied in a Venezuelan native which was classified as congenital by the results obtained from clinical and laboratory tests.

Methods

The coagulation tests were performed by the standard methods [15], and the plasma clot solubility in 5 M urea was done according to Losowsky *et al.* [8]. The defect of these patients being in the clot, it was thought that re-dissolving it with trypsin and studying it electrophoretically a difference might be observed when compared with the normal subjects. The following method was used: 0.5 ml of oxalated plasma mixed with 0.5 ml of 0.025 M of $CaCl_2$, incubated 30 min at 37°C; the clot was tapped loose and washed 3 times with NaCl 0.85%, and then trypsin digested with a drop of commercial trypsin (NBCo) 4 mg/ml at 37°C for 2 h, shaking gently at intervals 2 or 3 times; trypsin digestion was stopped adjusting to pH 6.4 with 0.5 N acetic acid, and centrifugated at 2000 rpm. Acetate cellulose electrophoresis of the supernatant liquid was made applying the sample 6 times.

hemoglobin indicates that the change from fetal to adult hemoglobin is markedly influenced by chronological age of the infant. Thus, change in hemoglobin seems to be more dependent on time than on the infant's weight, while for the A antigen the reverse is the case.

The significant partial correlation coefficient between strength of the A antigen and birth weight indicates also that weight is not merely an expression of size, but may provide information on the relative development of the infant. This has long been known to be valid in cases of prematurity. The present results reveal, however, that this is applicable even for infants resulting from pregnancies of the same duration.

Summary

Strength of the red cell A antigen and level of adult hemoglobin were found to be significantly correlated in newborn infants, even after correction for variation in birth weight and duration of gestation. *Furthermore*, strength of the A antigen was significantly higher in girls than in boys and much closer associated with birth weight than with duration of gestation. On the other hand, percentage of adult hemoglobin was somewhat closer associated with duration of gestation than with birth weight. The results indicated that relative development of the infant (expressed in birth weight) has a marked effect on the change in the A antigen, while the change in hemoglobin is more dependent on chronological age of the infant.

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Table I. Effect of the mother's plasma on the patient's clots. Incubation of the clots in 5M urea, expressed after B ad Duckert [14]

Subject studied	Correction power of plasma added *in vitro*							
	Pure	1 2	1 4	1 8	1 16	1 32	1 64	1 28
Control	++	+−	−−	−	−−	−	=	0
Mother	++	+−	−−		−	0	0	0

and indirect platelet count were all normal. The solubility of the recalcified plasma clot was demonstrated in 5M urea solution. Other hematologic and biochemical tests were normal, except the hemoglobin which was somewhat low 10.1 g 100 ml. Peripheral blood smear showed mild hypochromia of the red cell.

Physical therapy was instituted, consisting of moderate exercises and electrostimulation of the right anterior tibial muscles.

A year after she was discharged, the patient was hospitalized in coma in her home town and died within 24 h. Post mortem examination revealed intraparenchymatous cerebellar hemorrhage with rupture of the upper side of the right cerebellar lobe.

Electrophoresis of the recalcified plasma clot. The electrophoresis of the trypsin digested clot revealed, in addition to the albumin and fibrinogen 2 other fractions migrating ahead of the albumin. Other minor fractions, slower than albumin, were not identified (fig. 1). The patient's trypsin digested clot and that of her mother's, did not show difference when compared with the results obtained in normal subjects.

Family studies. The solubility of the plasma clot in 5M urea was done to the mother and maternal uncle, not finding abnormalities in either one. The capacity of correction of the mother's diluted plasma, done according to Jones *et al.* [3] was slower than the normal control (table I). This method has been used to determine heterozygous subjects.

Discussion

MacFarlane [16] estimated the probable incidence of FSF deficiency to be 0.1 × 10 inhabitants, which makes it one of the coagulation inborn errors of lowest frequency. Nevertheless, the possibility of mistaking it with the hemophilias should be kept in mind when dealing with hemophiloid states. The fact that to this writing only one case has been found in Venezuela – with a population of more than 9,500 000 – apparently confirms the reported low incidence of this disorder.

The genetic mechanisms of this condition has been considered as determined by a recessive autosomic gene although abnormal results found in the plasma of parents of some patients with congenital deficiency of FSF [1 5 13 14 17] allows the postulation of a possible

Table II. Clinical characteristics of the published

Case	Sex	Age years	Country	Parents consang.	Other family memb. studied deficients	non-deficients
1	M	7	Switzerland	yes	1 brother (case 2) 1 cousin	2 great-uncles 2 cousins
2	M	18m	Switzerland	yes	case 1	–
3	M		Finland	no	?	?
4	M	6	France	no	–	1 brother
5	F	2	Italy	yes	case 18	–
6	M	14m	France	no	mother[2]	–
7	M	4	France	yes	parents[3]	
8	F	52	Finland	yes		
9	M	26	USA	no	1 brother (case 10)	–
10	M	23	USA	no	case 9	
11	M	12	Canada	no	parents	
12	M	78	England	no	–	
13	M	4	England	no		
14	M	5	England	no	–	
15	M	24	Greece	?		
16	M	12	Denmark	no		parents
17	F	29	Israel	yes		
18	F	1	Italy	yes	1 sister (case 5	parents
19	M	3	USA	no	2 siblings parents grand-mother	maternal grand-father[3]
20	F		USA	no	parents 2 sisters 1 brother[3]	
21	F	20	Venezuela	no	mother	

Age at the time of diagnosis
Plasma clot was soluble in 5M urea
Partial deficiency of FSF demonstrated by titration

cases of congenital deficiency of FSF (Factor XIII)

Bleeding manifestations					Hemostat.		Authors
umbilical	ecchymoses	hematomas	dental	other	Cicatrizat.	eff. of transf.	
+	+	+	—	Post wounds, subdural hematoma	slow	yes	Duckert *et al.*, 1961 [1]
+	+	+	—			?	
—	+	—	—	post wounds			Ikkala and Nevanlinna, 196. [2]
+	+	+	—	post wounds		yes	Marcke, 1963 [4]
+	+	+	+	post wounds		yes	Mandelli 1963 [6]
+	+	+	—	otorrhagia, cerebro-meningeal		yes	Josso *et al.*, 1964 [5]
—	+	+	—	post wounds		yes	
+	—	—	—	hemoperitoneum, hemarthrosis		yes	Ikkala *et al.*, 1964 [3]
	+	+	+	epistaxis	slow	yes	Hampton *et al.*, 1965 [12]
?	+	+	+	epistaxis		yes	
—	+	+	—	hemarthrosis, subdural hematoma		yes	Barr and Delage, 1963 [14]
—	—	+	—	hemoperitoneum		yes	
+	+	+	—			yes	Losowsky *et al.*, 1963 [8]
+	+	+	—			yes	
—	—	—	—	post perineotomy post wounds	slow		Trevaldi *et al.*, 1965 [9]
—	+	+	—	post wounds, hemarthrosis, subdural hematoma	slow	yes	Amris and Ranek, 1965 [10]
+	+	+	+	abortions, hematuria	slow	yes	Fisher *et al.*, 1966 [11]
—	+	+	+	post wounds		yes	Ott... *et al.*, 1966 [7]
—	+	+	—	post wounds, hemarthrosis		yes	Britten, 1967 [15]
+	+	+	—	post wounds		yes	Bouhasin and Alta... 1968 [17]
+	+	+	—	post wounds, cerebellar hemorrhage		yes	this report

Partial deficiency of FSF demonstrated by thromboelastography
Platelets transamidase test
FSF levels

J. H. P. Jonxis and T. H. J. Huisman: ***A Laboratory Manual on Abnormal Haemoglobins.*** 2nd ed. Blackwell, Oxford/Edinbourgh 1968. 126 p. Price 30 s.

This manual was, as stated by the authors, 'primarily written for the medical pathologists and the laboratory workers receiving samples of blood for investigation but who have not made a special study in this field'. Indeed, the book is clearly and concisely written both in its initial part on clinical data and in the subsequent chapters on biochemical evaluation of abnormal hemoglobins. Much practical information reflects the vast personal experience of the authors, and many useful details are given which cannot be easily found in the original literature. The procedures include only few and important references to original publications which facilitates reading. In the reviewers opinion, there is too much referring from one chapter to another. On page 48 the authors state that 'the elution rates (on DEAE-Sephadex) are similar to those seen in DEAE cellulose chromatography' but in the corresponding section the reader again is referred to CM-chromatography concerning the same question. The reader is also supposed to be familiar with migration patterns of abnormal haemoglobins on electrophoresis (e.g. plates 3 and 4). The later chapters cannot be well understood without the complete reading of the earlier parts of the book. In general, the second edition of the Laboratory Manual on Abnormal Hemoglobins is a most welcome gift to every investigator interested in haemoglobinopathies.

E. Beck, *Basel*

H. S. Cox: **Medical Cyto-Technology.** Butterworth, London 1968. 65 p. Price 10 s.

The small monograph is concerned exclusively with exfoliative cytology and describes the current diagnostical methods including the technique for obtaining and preparing specimens of vaginal, cervical and endometrial smears, bronchial aspirates, gastric washings and ascites fluids. The staining techniques are reported and the characteristics of normal and malignant cells as well as the problems of cyto-screening are briefly discussed. Some recommendations for laboratory training are given at the end of the booklet, which offers a concise introduction in cytology for students, general pathologists and technicians and also stimulates more detailed studies necessary for the specialized cytologist.

H. R. Marti, *Aarau*

L. H. B. Walley and J. B. Robinson: **Human Haemoglobin Variants and their Identification.** Butterworth, London 1968. 56 p. Price 10 s.

The booklet contains in its first 15 pages a brief introduction in the field of haemoglobinopathies with summaries on the haemoglobin molecule, the genetic code, the abnormal haemoglobins, the thalassaemias, the Hb M variants and the inheritance and geographical distribution of the most frequent abnormalities. In these short chapters many details had to be omitted, only a small selection of abnormal haemoglobins are mentioned and the heterogeneity of thalassaemia is incompletely reported. In the main part of the booklet the current laboratory techniques are described with details necessary to perform the methods. Different techniques for detection of inclusion bodies, the sickle cell test, several methods of alkali denaturation, the technique of electrophoresis on cellulose acetate, starch gel and block, agar gel, polyacrylamide gel and paper, the ion exchange chromatography, the heat denaturation, Hb S solubility test and the stain for Hb F in red cells are given. This small volume is certainly a valuable help for pathologists and technicians.

H. R. Marti, *Aarau*

S. Seidl. **Die Thrombozytentransfusion.** Untersuchungen mit radioaktiv markierten Thrombozyten. Fischer, Stuttgart 1968. 119 S., 32 Abb. Preis DM 29.50.

Plättchentransfusionen haben im Laufe der letzten Jahre an Bedeutung gewonnen, da von klinischer Seite her, besonders im Zusammenhang mit der intensiveren zytostatischen Behandlung der Neoplasien, die Nachfrage stark gestiegen ist. Das vermehrte Interesse spiegelt sich auch in der rasch zunehmenden Flut der diesem Thema gewidmeten Literatur. Ein Überblick in der Art der vorliegenden Monographie kommt deshalb sicher einem allgemeinen Bedürfnis entgegen. Dabei hat der Autor sich die Sache nicht leicht gemacht: er begnügt sich nicht mit der Zusammenstellung der Literatur, sondern er hat versucht, die verschiedenen Ansichten und die noch offenen Fragen – und es sind deren viele – selbst zu überprüfen und mit besonderen Versuchsanordnungen einer Lösung näher zu bringen. Mit Recht legt der Autor für die Beurteilung der Funktionstüchtigkeit der Plättchen das Hauptgewicht auf die Überlebenszeit radioaktiv markierter Thrombozyten. Ein ausführlicher erster Teil ist der Methodik der Thrombozytenmarkierung (^{51}Cr und $DF^{32}P$) und allen damit zusammenhängenden Problemen gewidmet. Unter den für die Beurteilung der Thrombozytenfunktion herangezogenen in vitro-Methoden verdient vor allem die vom Autor erstmals auf Thrombozyten angewandte Zellelektrophorese Beachtung. Im Zusammenhang mit der Herstellung von Thrombozytenkonserven hat der Autor die wichtige Frage des geeignetsten Antikoagulans noch einmal aufgegriffen und mit Hilfe der in vitro-Methoden überprüft. Dabei konnte er die Überlegenheit der ACD-Lösungen mit niedrigem pH bestätigen. Zur Konservierung der Thrombozyten wird anhand eigener Versuche Stellung bezogen. Die Besprechung der klinischen Indikationen der Plättchentransfusion wird zum Anlass genommen, um Fragen des Thrombozytenumsatzes bei den verschiedenen Thrombopenieformen eingehend zu diskutieren. Im Abschnitt über die Thrombopenien als Folge eines vermehrten Verbrauchs finden sich interessante Beiträge zu den postoperativen Thrombopenien; die eigentlichen Verbrauchskoagulopathien, die regelmässig mit einer Thrombopenie einhergehen, finden jedoch keine Erwähnung. Der letzte Abschnitt ist immunologischen Aspekten der Thrombozytentransfusion gewidmet, wobei der Kreis so weit gezogen ist, dass fast das ganze Gebiet der Thrombozyten-Immunologie Berücksichtigung findet. Neben der Gegenüberstellung (und Wertung) der verschiedenen Methoden zum Antikörpernachweis scheinen uns die Untersuchungen zur Frage der Spezifität der Isoantikörper besonders wertvoll. Beachtung (und Nachprüfung) verdient sodann die Feststellung, dass das Serum von ITP-Patienten die elektrophoretische Beweglichkeit normaler Thrombozyten regelmässig zu vermindern vermochte. Der (hypothetische) »Autoantikörper« verhält sich offenbar in dieser Hinsicht anders als die Isoantikörper, welche in der Regel nicht zu einer Beeinflussung der elektrophoretischen Beweglichkeit führten. Im übrigen hat der Autor die Erfahrung anderer bestätigt, dass die Überlebenszeit der Plättchen den zur Zeit feinsten Hinweis auf das Vorliegen von Antikörpern zu geben vermag. Von praktischer Bedeutung ist schliesslich der Nachweis, dass Hämolysine oder Agglutinine des ABO-Systems mit erhöhtem Titer zum beschleunigten Abbau ABO-inkompatibler Plättchen führen.

Die Monographie vermittelt eine Fülle von höchst interessanten und wichtigen Daten aus dem Kapitel der Thrombozytenphysiologie und -pathologie. Bei der Vielschichtigkeit des Gebotenen mag der Titel des Buches sogar etwas zu bescheiden ausgefallen sein. Andererseits vermisst der praktisch orientierte Leser vielleicht eine verbindliche Empfehlung, wie im Einzelfall für die Thrombozytentransfusion vorzugehen sei, z.B. in Bezug auf Wahl des Präparates (PRP oder Konzentrat), technische Durchführung der Konzentration unter Berücksichtigung aller diskutierten Erkenntnisse, Anzahl der benötigten Spender, Möglichkeiten der Thrombopherese, usw. Das Buch wird aber von jedem, der sich mit den Thrombozyten beschäftigt und der sich über die Möglichkeiten der Plättchentransfusion orientieren möchte, mit Gewinn gelesen werden. Die klare Gliederung erleichtert die Lektüre, wobei wir die am Ende jedes Abschnittes gegebene Zusammenfassung als besonders angenehm hervorheben möchten.

U. Bucher, *Bern*

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